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14. ABSTRACT:
The research supported by the Department of Defense Breast Cancer Research Program (BCRP) studies whether the polyphosphate survival function is conserved between prokaryotes and eukaryotes and specifically whether polyphosphates are involved in the increased survival of breast cancer cells. While in the process of developing modified MCF-7 breast cancer cells, an exciting opportunity arose to investigate polyphosphates and their effect on DNA damage response. It is well documented that DNA damaging agents can cause genomic instability and lead to various forms of cancer including breast cancer. Without proper cell cycle checkpoints that trigger repair of the damage or induction of apoptosis, genetic mutations can be propagated, possibly initiating tumorigenesis. Functional analysis of DNA damage response, cell cycle checkpoints which involve BRCA1, genome integrity, and tumor evolution will build the knowledge of the mechanisms involved in breast cancer. The principle investigator ceased this opportunity to study polyphosphates and DNA damage response with respect to cell survival and DNA repair mutagenesis and the DNA damage response (SOS response) which follows extensive DNA damage. It has been discovered that: Loss of ability to synthesize polyphosphates compromises cellular survival after DNA damage; This effect is not exclusive to a specific DNA damaging agent; This loss of survival is not directly linked to the general stress response pathway; This compromised survival is independent of cellular growth phase or growth rate; Polyphosphates levels increase transiently after DNA damage; Elevated polyphosphate levels are not dependent on the SOS genes recA, dinB, or umuDC; The ability to synthesize polyphosphates influences Pol IV activity.

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INTRODUCTION

Cellular polyphosphates (polyP), dynamic long linear polymers of orthophosphate linked by high energy phosphoanhydride bonds, are ubiquitous, being found in every organism and tissue examined. Polyphosphates can be synthetically generated by dehydration of P_i at elevated temperatures but in living cells these anionic polymers are very dynamic, being continuously synthesized and degraded by metabolic enzymes. They are found in chains of tens to hundreds of phosphate residues in length in a variety of cellular compartments (Schroder, 1999). Numerous functions have been proposed based on their physical properties. These include: ATP substitute and energy source, reservoir of orthophosphates, chelator of metal ions, buffer against alkali, channel for DNA entry, and regulator of stress and survival with prokaryotic model systems (Schroder, 1999). While little is known about the function of these polymers, emerging studies indicate that polyphosphates promote cell survival by participating in stress response pathways, with reduced polyphosphate levels compromising how cells respond to and survive environmental stresses. The research supported by the Department of Defense Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP) studies whether the polyphosphate survival function is conserved between prokaryotes and eukaryotes and specifically whether polyphosphates are involved in the increased survival of breast cancer cells.

BODY

(Large portions of the body of this report is adapted from doctoral thesis: Haakenson,C. (2007) "Polyphosphates Enhance Cell Survival Following DNA Damage by Influencing SOS Induced DNA Repair Mechanisms", Unpublished Doctoral Dissertation, Georgetown University)

During the duration of the predoctoral training grant W81XWH-04-1-0379, the role of cellular polyphosphates was examined. Polyphosphates must be significant in basic cellular functions since these high energy molecules are ubiquitously present in every prokaryote and eukaryote examined. It is easy to argue that if polyphosphates did not have a vital cellular function, selective pressures would have prevented their conservation throughout evolution.

An understanding of the role of polyphosphates in cellular physiology began to emerge during the early 1990's with the identification of the genes and corresponding enzymes involved in polyP synthesis and degradation. The biosynthetic enzyme, polyphosphate kinase (PPK) has been purified from *Escherichia coli* (*E. coli)* (Akiyama *et al.*, 1992), as have an exopolyphosphatase (PPX) and endopolyphosphatase (PPN), which also have been isolated from *S. cerevisiae*. Cells with genetically altered levels of polyP have been created by deleting or overexpressing the genes encoding PPK and PPX (Akiyama *et al.*, 1993; Kumble *et al.*, 1996; Wurst *et al.*, 1995). The purified enzymes have also proven to be invaluable tools for developing sensitive and reliable assays to measure polyP levels in different organisms and tissues (Ault-Riche *et al.*, 1998).

In prokaryotes, accumulating data indicate that polyP promotes cell survival. Reduced polyP levels compromise how the cells respond to and survive environmental challenges, such as nutrient deprivation, heat shock, phosphate deficiency, oxidative stress, and osmotic challenge. Relatedly, polyP levels have been observed to transiently increase following exposure to such

conditions. While these general characteristics regarding polyP have been determined for micro-organisms, few studies have examined polyphosphate concentration and location in mammalian cells. Kumble and Kornberg found levels of 25 to 120 μM (in terms of P_i residues) in rodent tissues (brain, heart, kidneys, liver, and lungs) and specific subcellular fractions (nuclei, mitochondria, plasma membranes, and microsomes). The synthesis of polyphosphate from P_i by fibroblasts, T-cells, kidney, and adrenal cell lines attained levels of excess of 10 pmol per mg of cell protein per hour. Further tests showed the turnover of polyphosphate varied from one hour in adrenal cells to over four hours in fibroblast cells. They concluded that the ubiquity of polyphosphate and variations in its polymer length, location, and metabolism are indicative of a multiplicity of functions for this polymer in mammalian systems (Kumble and Kornberg, 1995).

A direct relation between breast cancer and polyphosphate has been observed in one study. MCF-7 cells depleted of polyphosphate by overproduction of scPPX did not grow in serum-free media, while MCF-7 cells without overproduction of PPX grew normally to confluence. Polyphosphate was also found to stimulate M-Tor, but not other related DNA protein kinases. Interestingly, M-Tor is part of a signaling pathway that activates anti-apoptosis proteins (Wang *et al.*, 2003). The research, described in this summary report and supported by the Department of Defense, has allowed the relationship between breast cancer and polyphosphates to be further studied while contributing to the education and training of a doctoral student.

A protocol, which gathered polyP from *E. coli* cells, was modified and optimized to be used on breast cancer cells. In order to gather the polyP from the breast cancer cells, the cell pellet was thoroughly lysed and treated with RNase, DNase, Proteinase K and SDS to degrade the nucleic acid and disrupt protein interactions. Due to the significantly larger quantity of DNA in the breast cancer cells versus bacterial cells, the RNA and DNA needed to be thoroughly degraded prior to binding the polyP to the silica slurry. Any non-degraded nucleic acid would also bind to the silica, possibly competing out the polyP from binding. The lysate was combined with a slurry of a silica compound which binds with anionic chains. Once the polyP is washed and eluted from the silica, it is transferred into ATP through the PPK reverse reaction. One ATP is produced from each phosphate in the polyP chain. ATP can then be measured by the quantity of light produced in a luciferase reaction.

A plasmid was constructed containing a copy of the *S. cerevisiae* exopolyphosphatase (scPPX) gene behind a constitutively active CMV promoter. scPPX rapidly degrades polyP by processively liberating the end phosphate moiety from the polymer. Therefore, the addition of extra copies of scPPX into a cell will decrease its polyphosphate levels. The constructed plasmid was stably transfected into breast cancer cell lines MCF-7. Differences in polyphosphate levels between these new cells and the original, unmodified breast cancer cell were not yet confirmed.

While in the process of developing stable transfections of (PPX1) from *S. cerevisae* into MCF-7 breast cancer cells, an exciting opportunity arose to investigate polyphosphates and their effect on DNA damage response. It is well documented that DNA damaging agents can cause genomic instability and lead to various forms of cancer including breast cancer. Without proper cell cycle checkpoints that trigger repair of the damage or induction of apoptosis, genetic mutations can be propagated, possibly initiating tumorigenesis. Functional analysis of DNA damage response, cell cycle checkpoints which involve BRCA1, genome integrity, and tumor evolution will build the knowledge of the mechanisms involved in breast cancer (Deng, 2006)

The principle investigator modified the aims of this study to cease this opportunity to study polyphosphates and DNA damage response with respect to cell survival and DNA repair mutagenesis. A simplified model system was used to study this relationship. Prokaryotes have much simpler cell structure and are the root of the evolutionary development of higher organisms. By first understanding the functionality in this model system, the future research of polyphosphates in breast cancer cells can be more directed and more efficient. Therefore, *Escherchia coli* have been used to study polyphosphates effect on DNA damage and the DNA damage response (SOS response) which follows extensive DNA damage.

E. coli SOS Response

Organisms are continually exposed to physical and chemical agents (e.g., ultraviolet light, carcinogens, and mutagens) that damage DNA. Their survival depends on a DNA repair mechanism to restore normal cellular functions and ensure survival. There are many repair mechanisms characterized that correctly fix the damage DNA prior to complications with DNA replication. These include base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), or recombination repair. When the DNA damage is extensive, and these mechanisms are unable to fully repair the DNA, a survival response is triggered which induces all forms of repair, including error prone repair, as quickly as possible. Changes in *E. coli* gene expression in response to DNA damage were first described in the early 1970s and were collectively termed the SOS response, after the international distress signal (Friedberg, 2006). The SOS response is the best characterized DNA repair system is the bacterial system, which comprises a set of genes that are coordinately induced by excessive DNA damage to affect enhanced DNA repair capacity, enhanced mutagenesis, and inhibition of cell division (Au *et al*., 2005; Friedberg, 1985; Friedberg, 1989).

The SOS response induces more than 40+ SOS genes (Courcelle *et al.*, 2001) and many of the gene products are proteins involved in the non-error prone repair mechanism listed above. The induction increases the concentration of these proteins to increase the number of repaired DNA sites. Also, among the SOS genes induced are those encoding the specialized DNA polymerases, Pols II, IV and V (Goodman, 2002). The SOS polymerases catalyze translesion synthesis (TLS) by replacing a replicative Pol III that stalls when encountering a damaged template base. Once past the damage site, Pol III takes over to restart normal DNA replication. TLS, which results in mutations targeted to the sites of DNA damage, appears to be the biological basis of SOS mutagenesis.

The molecular model for the SOS systems has been continuously supplemented throughout the years with more experimental research providing mechanistic details. The most current model is depicted in figure 1 which is modified from Goodman (Goodman, 2002). The key controllers of SOS induction are the RecA and LexA proteins. Under normal circumstances, the LexA protein represses SOS gene expression by binding to a specific DNA sequence, or SOS box, overlapping the promoter region for SOS-induced genes. RecA is the SOS system inducer. RecA responds to the cellular distress call. The distress occurs when single stranded DNA (ssDNA) is formed from blockages during DNA replication due to the cell's failure to repair and replicate damaged DNA. The excessive ssDNA signals that the genome is in disarray and the cell will soon die if

action is not taken. Therefore the SOS system, when fully induced, functions as a last ditch attempt to endure substantial DNA damage that has inhibited most, if not all, replication forks.

RecA's role is not only to recognize ssDNA forming nucleoprotein filaments and induce the SOS response but it is also engaged in two other distinct roles in the cell. Its principal role is to catalyze DNA strand pairing, leading to homologous recombination (Kuzminov, 1999). The third role is also part of the SOS mechanism after the induction. RecA is necessary for activating Pol V, a polymerase involved in SOS mutagenesis (Walker, 1984; Witkin, 1976).

During SOS induction, RecA is activated and induces LexA repressor autocleavage, thereby destroying its ability to bind an inhibitor transcriptional SOS box. The damage-inducible (din) SOS genes are transcriptionally up regulated as a result, leading to a variety of physiological changes collectively described as the SOS response. Once there is no longer single stranded DNA, RecA is no longer activated and replenished intracellular LexA once again binds SOS boxes, repressing the SOS genes. It is found that *recA* and *lexA* gene promoters are LexA-inhibited and RecA plays in its own induction (Little *et al*., 1981). This provides a tight control over SOS induction and SOS inhibition. This systemic shutdown has been estimated to occur roughly 1 hour post-induction.

SOS genes are defined by their RecA-dependent induction in response to DNA damage, and their having at least one LexA binding site, or SOS Box, in their promoter regions. Each gene is induced at different times depending on their number of SOS boxes and the degree of sequence similarity for the LexA inhibitor. Genes with weak LexA binding sites are induced first in the SOS response while genes with tight binding sites and multiple binding sites are not induced until the end of the hour induction. The delayed induction of genes results in two phase response (Defais and Devoret, 2005; Shinagawa, 1996; Smith and Walker, 1998; Sommer *et al.*, 1998; Walker *et al.*, 2000).

The first phase is a largely accurate phase dominated by accurate repair processes such as excision repair and recombinational DNA repair. Error-free, classical repair mechanisms (NER, BER, and MMR) are induced to repair sites throughout the damaged genome prior to replication forks reaching these sites. If these damaged sites are reached by the replication fork, the forks stall or collapse. Recombinational repair systems rebuild the fork structure and repair the damage before transferring the fork back to Pol III for high fidelity and processive replication. The fork will collapse again if it reaches additional damage downstream. If there is extensive damage and the forks are caused to stall repeatedly, phase two of the SOS response begins, the mutagenic phase.

Phase two induces the error-prone polymerases, Pol IV and Pol V, which participate in translesion repair. Error-prone repair allows replication to continue through the DNA lesions by placing a "best guess" nucleotide at the damaged site. In exchange for increased survival, the cell pays a cost of an elevated mutation rate resulting from this translesion synthesis. Phase two may be successful in repairing all of the lesions but the cell still may not survive due to the genetic mutations. This explains the need for the two phase response. The cell hopes to recover from the damage in phase one to prevent mutagenesis.

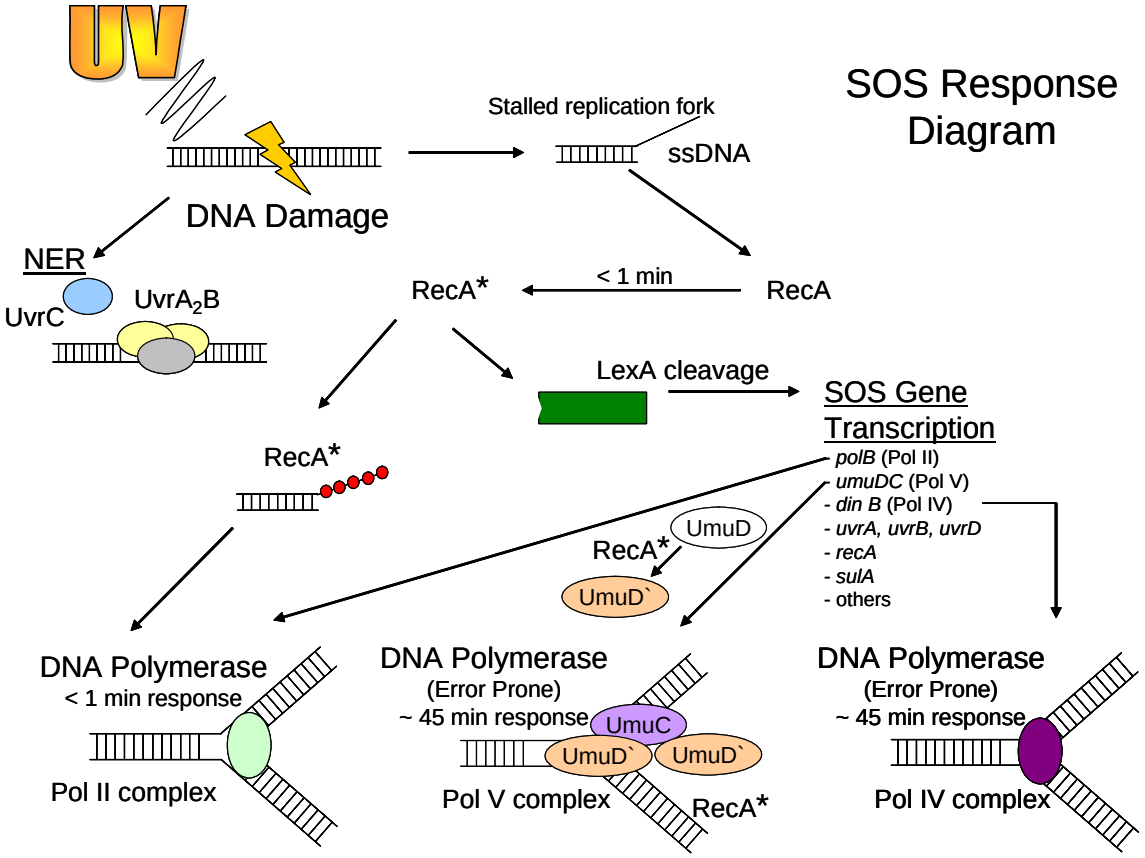


Figure 1 – SOS Response

The diagram above depicts the SOS response mechanism. Based upon (Goodman, 2002). The SOS System is activated in the presence of DNA damage. In the absence of damage, LexA represses SOS genes via binding to their operator sequences, or SOS Boxes. DNA damage generates ssDNA most typically through stalled replication forks, which stimulates RecA activation (<1 min after DNA damage) upon binding to form a nucleoprotein filament (RecA*). RecA* induces the autocleavage of LexA, thereby derepressing the SOS genes and allowing their transcription. Differing LexA binding affinities to distinctive operon sequences of the various proteins regulate the protein induction in a time-dependent manner. In a similar reaction taking place on RecA*, UmuD is cleaved to generate the active carboxy-terminal fragment UmuD'. Two molecules of UmuD' combine with one UmuC to form Pol V. *polB*, encoding Pol II, is expressed early (<1 min post DNA damage) and is involved in error-free replication restart. Pol V and Pol IV appear much later (45 min post DNA damage). When DNA damage has been repaired, the ssDNA signal is eradicated and RecA* is deactivated. Renewed LexA repression shuts off the system, and the cell returns to its normal state.

Error-Prone Polymerases

SOS mutagenesis is generated in *E. coli* from the Y-family polymerases, Pol IV (DinB) and Pol V ($UmuD'_2C$). These polymerases carry out poor replication accuracy which gives them the ability to replicate through DNA damage. In a 2002 review by Myron Goodman, error-prone polymerases were defined by having one or more of the following properties: (Goodman, 2002)

(a) an ability to copy damaged DNA with high efficiency, either alone or in the presence of accessory proteins

(b) poor accuracy in nucleotide incorporation with base substitution error frequencies of 10^1 to 10^3

(c) a tendency to form base mispairs rather than correct Watson-Crick base pairs

(d) a propensity to catalyze incorporation using aberrant DNA primer ends, including base mismatches, misaligned primer-template, and DNA damage sites.

The error-prone polymerases are able to replicate DNA past a lesion despite a missing template by randomly incorporating nucleotides. TLS accounts for the high mutation rate seen in cells that have undergone massive DNA damage, but these potentially deleterious mutations are preferable to the otherwise inevitable cell death.

The variances of structure between polymerases give them their difference in functionality. Non-error-prone polymerases have high fidelity by imposing active-site geometric constraints strongly favoring incorporation of Watson-Crick base pairs and by proofreading/removing mismatched nucleotides that slipped through the structural constraints (Goodman, 2002). Crystallographic data suggest that the active cleft architectures of error-prone polymerases are much less restrictive, accommodating non-Watson-Crick pairs along with distorted primer/template DNA caused by the presence of damaged DNA bases (Ling *et al.*, 2001; Silvian *et al.*, 2001; Trincao *et al.*, 2001; Zhou *et al.*, 2001).

Both error-prone polymerases and error-free polymerases require the β-clamp protein to help bind and remain bound to DNA. The β-clamp is a ring-shaped dimer that encircles DNA and contains a hydrophobic pocket located on each β monomer surface (Dalrymple *et al.*, 2001; Lopez de Saro and O'Donnell, 2001). Through these hydrophobic pockets it tethers to polymerases as a pentapeptide motif, QL(SD)LF, thereby ensuring strong polymerase-DNA binding and increasing polymerase processivity. For example, Pol III increases its processivity the β-clamp from about 10 nucleotides to several thousand nucleotides per template binding event (Kelman and O'Donnell, 1995; Kong *et al.*, 1992; Oakley *et al.*, 2003). All of the polymerases, including SOS induced polymerases, Pol II, IV, and V utilize the β-clamp. Pol IV, which by itself binds very poorly to both DNA and dNTP substrates, also exhibits a large increase in dNTP binding affinity in the presence of the β-clamp (Bertram *et al.*, 2004).

The β-clamp presumably acts as a platform on which TLS polymerases switch with Pol III when the replicative polymerase becomes stalled at a damaged template base. Since the polymerases appear to interact with the same hydrophobic pocket on the β-monomer and since the Pol III core does not easily dissociate from the β-clamp when loaded, various models have been proposed (Johanson and McHenry, 1982; McHenry, 1991). Either the different

polymerases compete for the hydrophobic binding site on the β-clamp switching back and forth between polymerases (Burnouf *et al.*, 2004; Lopez de Saro *et al.*, 2003) or the dimeric β-clamp with two hydrophobic pockets acts as a toolbelt allowing two polymerases to be localized to the replication fork simultaneously. Both models have been witnessed in *in vitro* assays. Fujii and Fuchs observed sequential usage of the β-clamp by Pol III and Pol V (Fujii and Fuchs, 2004), while Indiani *et al* identified a ternary complex with Pol III, Pol IV and the β-clamp (Indiani *et al.*, 2005).

Competitive versus tool-belt switching may not be mutually exclusive mechanisms, and each might operate under different sets of circumstances, such as whether cells are dividing in log or stationary phase or perhaps not dividing. It is known, for example, that TLS bypass efficiencies *in vivo* depend on the identity of the lesion and the polymerase used to copy it. For example, Pol V repairs abasic sites, TT dimers, and 6-4 photoproducts much more efficiently than either Pol II or Pol IV (Tang *et al.*, 2000), whereas bulky adducts, such as acetyl aminofluorenes and benzopyrenes, are better substrates for Pols II and IV (Shen *et al.*, 2002; Wagner *et al.*, 2002). It has also been shown that Pol V is almost entirely responsible for SOS mutations occurring on chromosomal DNA in exponentially dividing cells (Kato and Shinoura, 1977; Steinborn, 1978), whereas Pol IV appears not to mutate the chromosome during exponential growth (Brotcorne-Lannoye and Maenhaut-Michel, 1986; Kuban *et al.*, 2004; Wolff *et al.*, 2004). There is most probably and balance between the two models allowing for both factors, the accessibility of the polymerases versus the lesion identity.

The polymerase availability fluctuates based on cellular growth phase and SOS induction. In the absence of SOS induction, the copy number of Pol V is undetectable (<15 molecules/cell). Once induced, approximately 200 Pol V protein complexes are formed which is still a low number compared to the other Y-family polymerase, Pol IV. An uninduced cell contains 250 copies of Pol IV and increases 10-fold when induced by SOS to 2,500. Pol II, the third SOS inducible polymerase increases 7-fold from 50 to 350 copies. Each of these polymerases can be compared to Pol III, polymerase involved in chromosome replication. A cell only has 20 copies of Pol III and in not induced by SOS (Nohmi, 2006).

Pol V

In E. *coli,* UmuC and UmuD form DNA Polymerase V through a RecA-dependent mechanism. As described above, the SOS response is triggered when replication forks stalled at DNA damage resulting in ssDNA. RecA is activated in the presence of ssDNA and catalyzes the autocleavage of LexA releasing the LexA inhibitor from the promoters of SOS induced genes. One of the induced genes is *umuDC* which produces a heterotrimer protein, Pol V, composed of one UmuC bound to two UmuD' molecules (UmuD'$_2$C). UmuD’ is an activated segment of UmuD created through facilitation by RecA in a LexA-like autocleavage, Figure 2. RecA also participates in the activity of the Pol V holoenzyme at the replication fork (Schlacher *et al.*, 2006b).

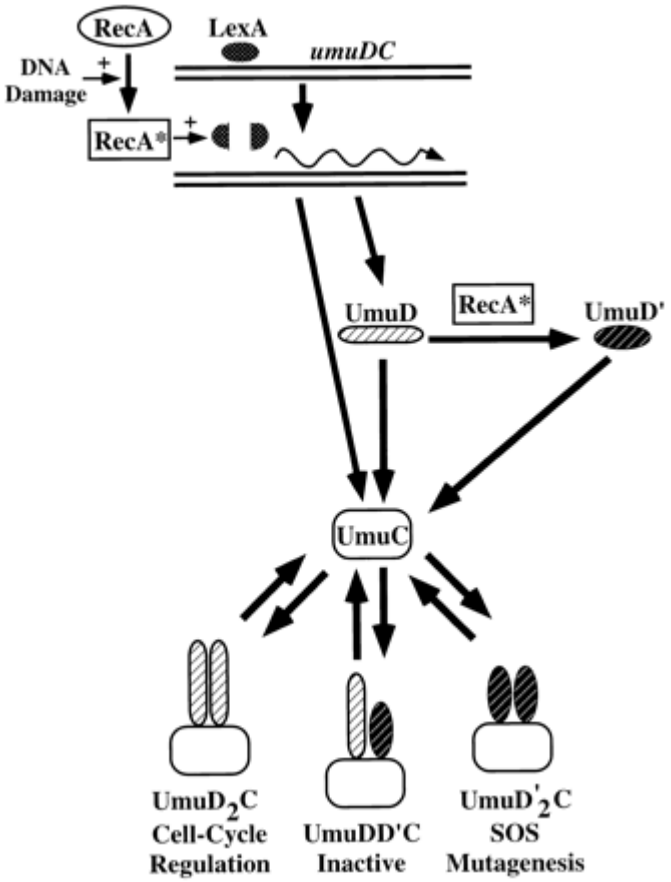


Figure 2 – Regulation of the umuDC operon by RecA and LexA

DNA damage generates a signal that converts RecA to RecA*. RecA* mediates the cleavage of the LexA repressor that results in the induction of the umuDC operon as well as the rest of the SOS response genes. RecA* can also mediate the processing of UmuD to the shortened UmuD' molecule. UmuD and UmuD' can interact with UmuC in a variety of combinations with the Umu(D')2C complex being active in TLS. Adapted from (Smith and Walker, 1998).

A recent study determined Pol V catalyzed TLS occurs only when RecA nucleoprotein filaments assemble on separate, ssDNA, molecules in *trans*. This contradicted the previous cowcatcher model that had RecA binding the ssDNA in front of the replication assembly and were knocked off the nucleic acid as the replication progressed (Schlacher *et al.*, 2006a). Figure 3 depicts this new model.

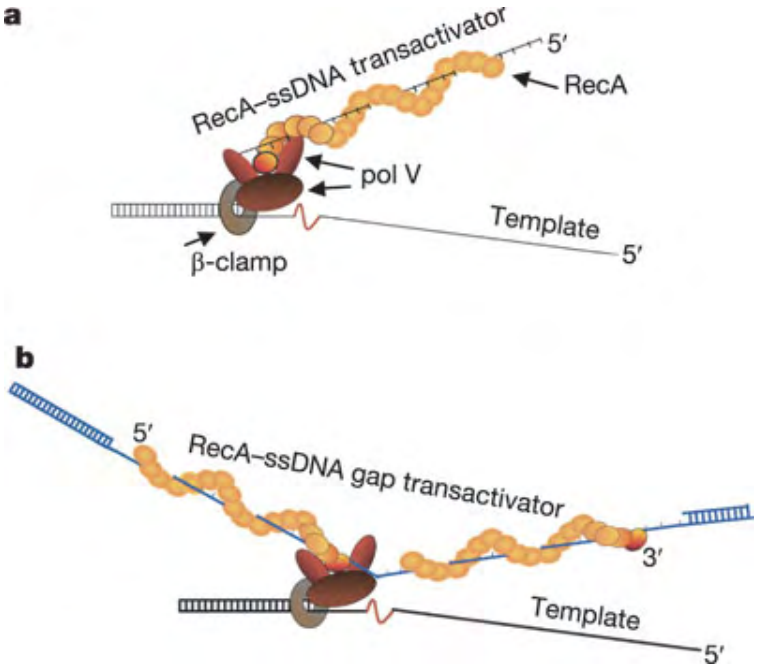


Figure 3 – RecA*-Pol V active complex

RecA*–ssDNA is required for Pol V activity to repair DNA. The essential feature of the transactivation model is that a RecA*–DNA complex is formed on ssDNA that is not being copied by Pol V. If RecA* were to bind instead to the template strand, cis to Pol V, then TLS would be blocked. A variation on the model shows how transactivation of Pol V might occur by a RecA* filament formed in gapped DNA. Adapted from (Schlacher *et al.*, 2006a).

Pol IV

As described above, Pol IV is a member of the error-prone Y-family polymerase which is capable of synthesizing DNA past DNA lesions. Less is known about the cellular functions of Pol IV compared to Pol V (Goodman, 2002). The 351 amino acid protein is synthesized from the 1056 bp *dinB* gene located on the *E. coli* genome at 5.4 min. Approximately 250 copies of the protein exist in the cell prior to induction suggesting additional roles outside of SOS and RpoS inductions. A ten fold induction (250 to 2500 copies) occurs by either the RpoS transcription factor after long term stationary phase independent of LexA inactivation (Foster, 2005), or by the SOS response dependent of LexA cleavage through RecA.

The ability of Pol IV to replicate past damaged DNA is limited and depends on lesion, sequence context, and growth conditions (Foster, 2005). Research indicates Pol IV is not required for SOS lesion-targeted UV mutagenesis and Pol IV prefers DNA lesions of non-UV origin, for example, benzo(*a*)pyrene-guanine adducts (Shen *et al.*, 2002) and 4-nitroquinoline *N*-oxide (Kim *et al.*, 2001). However, it is noted the data analyzed for cell survival following

various DNA damaging agents were found to use cultures grown to log phase growth or cultures grown in rich media.

Pol IV replication requires the β-clamp, as do the other DNA polymerases, but it does not need any other cofactors, such as the cofactor RecA needed for Pol V activity. Since the copy number of Pol IV exceeds Pol V nearly 10 fold, Pol IV may become the dominant DNA polymerase competing for the β-clamp. Nevertheless, since there is a preference for Pol V by DNA damaged with thiamine dimmers formed by UV, it is not fully understood how the polymerase is selected for TLS, suggesting a sensitive balance between polymerase copy number, dissociation constant with the β-clamp, lesion type, and growth conditions.

The core function associated with Pol IV is adaptive mutagenesis. Pol IV provides 50-80% of the adaptive mutations that occur in bacteria under non-lethal selection, starving non-dividing cells (Foster, 2000). Adaptive mutagenesis, originally called directed mutagenesis, is the process that produces advantageous mutations that arise due to selective pressure. When bacteria are subjected to non-lethal selection, mutations arise to try to relieve this selective pressure (Cairns *et al.*, 1988). Bacteria in the population that generate beneficial mutations gain the functional advantage over the surrounding bacteria which do not obtain the functional improvement.

A relationship between Pol IV and polyphosphates has previously been suggested (Stumpf and Foster, 2005). While looking at adaptive mutagenesis, it was discovered that interruption of the *ppk* gene caused a decreased level of mutations. This supports the theory that polyphosphates, or PPK, assist directly or indirectly in the activity of the Pol IV replisome. When cells were grown to stationary phase to induce adaptive mutagenesis, the 10 fold increase of Pol IV produced enough molecules to compete with other polymerases for the β-clamp and the replication fork. Foster and Stumpf did not find a difference in the Pol IV levels in the strain with an interrupted *ppk* gene proving the difference in Pol IV activity in the PPK- strains is not due to copy number of the polymerase (Stumpf and Foster, 2005). It was not determined how polyphosphates or PPK affect mutagenesis nor was it studied if polyphosphates or PPK affect mutagenesis under different conditions.

Polyphosphates and the SOS Response

Limited information links polyphosphates to the SOS response. As mentioned above, polyphosphates or PPK, directly or indirectly, regulates DNA polymerase activity, or fidelity, for Pol IV during adaptive mutagenesis (Stumpf and Foster, 2005). However, this has not yet been seen in Pol IV activity following SOS induction. Other evidence that links polyphosphates and the SOS response involves the *recA* and *umuDC* genes, which code for two SOS-induced proteins. Normally these genes have increased expression during the SOS response, but cells with reduced quantity of polyphosphates did not have the same degree of increased level of *recA* and *umuDC* expression after significant DNA damage (Tsutsumi et al., 2000). This information suggests a link between polyphosphates and the SOS response but it is unclear if it a direct influence on the SOS response or a secondary effect due to polyphosphates influence on the general stress response.

Polyphosphates were found to assist cell survival after an environmental stress, activating the general stress response (Rao and Kornberg, 1999). It has been seen that, within this response,

polyphosphates affect transcription of survival genes (Shiba *et al.*, 1997), Lon protein degradation (Kuroda *et al.*, 2001), stringent response (Rao *et al.*, 1998) and adaptive mutagenesis (Stumpf and Foster, 2005). A cellular compound that affects that many components of the general stress response is likely to affect cell survival after other environmental stresses, such as DNA damage activating the SOS response.

Polyphosphates have been reported to bind to the Lon protein in the ATPase domain of the protein. It also binds ribosomal proteins to facilitate the interaction of the Lon proteins and the ribosomal proteins for degradation (Kuroda, 2006). This is not the only report of polyphosphate-protein contact. Polyphosphates also bind ribosomal proteins involved in protein translation (McInerney *et al.*, 2006). It is suggested that polyphosphates are able to bind some proteins that also bind nucleic acids. A protein that binds nucleic acid on the phosphodiester backbone may also bind to polyphosphates. The majority of the proteins involved in DNA repair and the SOS response bind nucleic acids. Therefore, polyphosphates may have an effect on these specific proteins.

As mentioned in the introduction, error-prone polymerase IV is induced by the SOS response and is a major component of adaptive mutagenesis. In 2005, it was reported that cells without PPK had reduced adaptive mutagenesis activity by Pol IV (Stumpf and Foster, 2005). If polyphosphates already affect Pol IV in adaptive mutagenesis, it is likely to affect Pol IV in DNA repair.

The majority of polyphosphate research has identified associations with polyphosphates, but has not identified the physical mechanism on how polyphosphates cause observed cellular changes. In fact, most research can not point to the location within the functional pathway where polyphosphates interact. Further knowledge of the mechanism by which polyphosphates act within a pathway would increase the understanding of the molecular pathway, and help identify potential mechanisms in other cellular functions.

In this study, the relationship between polyphosphates and DNA damage was affirmed through cell survival assays and polyphosphate concentration measurements. Once it was shown that polyphosphates had an impact on cell survival through the SOS response, the relationships between polyphosphates and key SOS proteins were analyzed using epistatic assays to address cellular polyphosphates' participation in environmental stress survival mechanisms following U.V. radiation by stimulating response protein effectiveness, which results in enhanced survival. The results from this work will help to determine additional functions of polyphosphates, both specifically for DNA repair and to gain some understanding of other potential polyphosphate functions. These potential functions include functions within eukaryote systems, especially in relation to breast cancer.

RESULTS

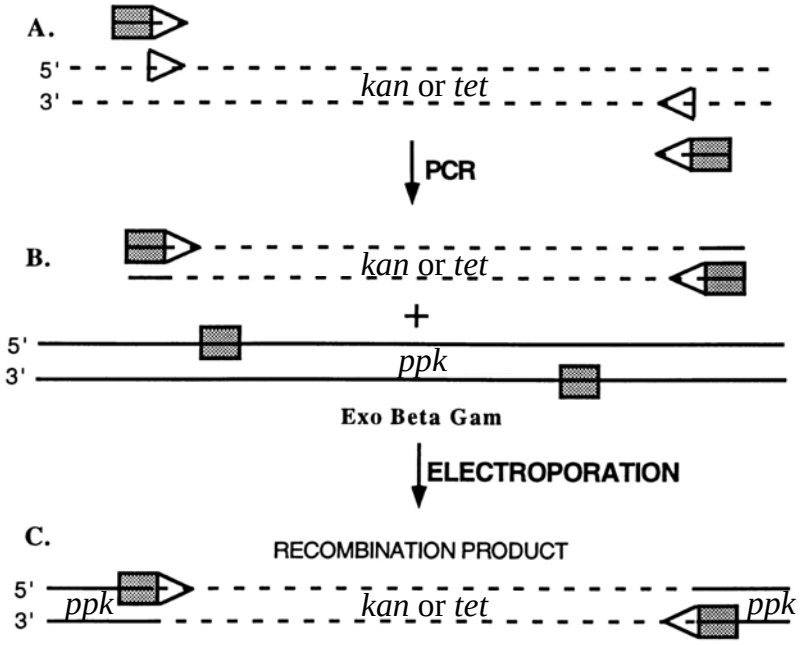
Strain Construction

Before *E. coli* could be analyzed for survival differences between strains with and without PPK and key SOS proteins, the strains needed to be genetically constructed. A total of 18 strains were constructed and utilized in the survival assays. Table 1 contains a complete list of all

strains and plasmids. The majority of the strains were developed through P1 transduction to move a mutated gene from one strain to the desired host. Furthermore, the two *ppk* interrupted strains used the RED cloning methodology to enter the selectable marker, either *tet* or *kan*, into the middle of the *ppk* gene.

Strain Construction for ppk Interruptions

As mentioned above, strains were developed that contained disrupted *ppk*. Ultimately, the *ppk* gene in MG1655 host contained a *kan* insert or a *tet* insert. To produce these strains, the RED cloning procedure was used to interrupt *ppk* in the bacterial genome (Yu *et al.*, 2000). The RED cloning procedure is described in the methods. Primers for the selectable markers were designed with approximately 42 bp overhangs which have homology to sequences in the *ppk* gene. Figure 4 depicts the PCR design and gives the primer sequences. The PCR products were electroporated into a DY330, an *E. coli* strain which contained a λ prophage harboring the recombination genes *exo*, *bet*, and *gam* under control of a temperature-sensitive λ cI-repressor.



Modified from Yu, Daiguan et al. (2000) Proc. Natl. Acad. Sci. USA 97, 5978-5983

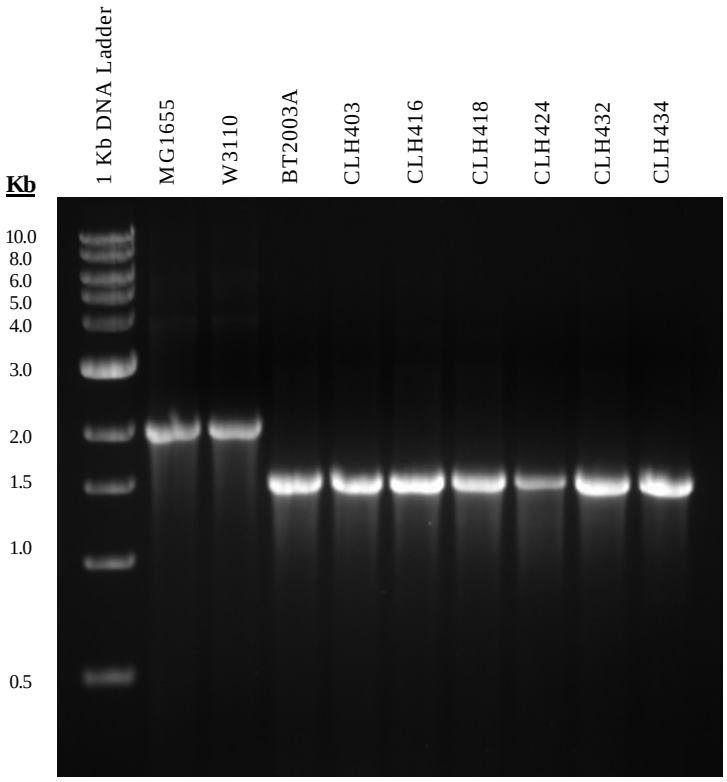
Selectable Marker	5' Primer	3' Primer
kan	gga tga gtt tcc tgg gga tct att cca ata acc ttg atg agt tct ata aa g cca cgt tgt gtc tca aaaq tct c	ccg tat cgc tga aca ata tgt cga tga tgt cca gca ccc gct tca ggc gct gag gtc tgc ctc gtg aag
tet	gtt tcc tgg gga tct att cca ata acc ttg atg agt tct ata aac aag agg gtc att ata ttt cg	ccg tat cgc tga aca ata tgt cga tga tgt cca gca ccc gtt act cga cat ctt ggt tac cg

Figure 4 – ΔPPK Strain Construction

The illustration describes the procedure to insert a selectable marker into a gene within the genome. The primers listed contain sequence similarity with the *ppk* gene and primers for either *kan* or *tet*, underlined.

After *ppk* was interrupted in the background strain DY330, it was moved into the MG1655 host using P1 transduction as described the methods. To screen and verify the strains that were developed, PCR was used to amplify the genome and compare the size of *ppk* fragments. Figure 5 shows the PCR screen for all *ppk* interrupted strains.

A.



B.

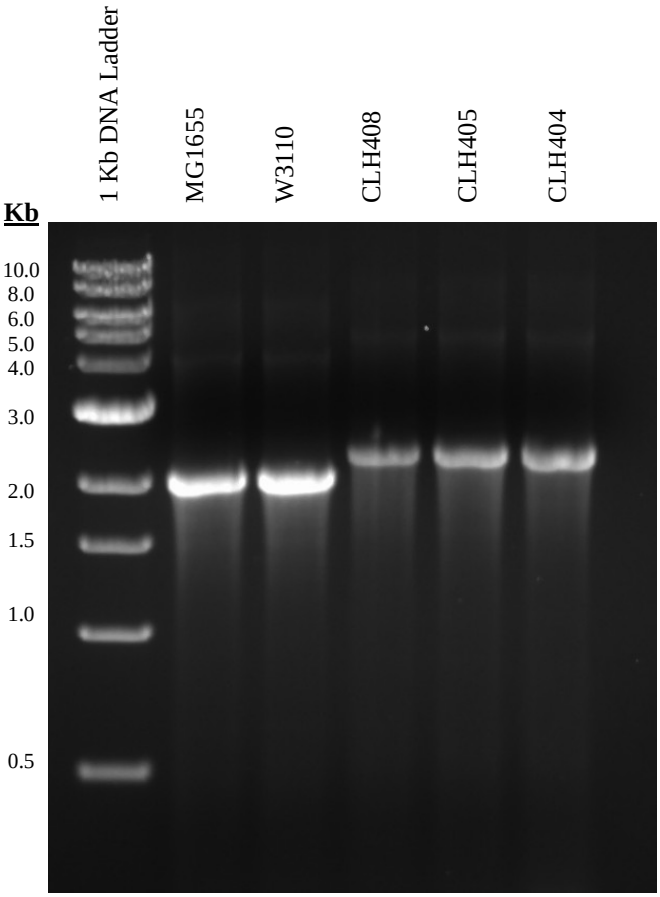


Figure 5 – Has the *ppk* gene been interrupted in the strains?

1% agarose gels with ethidium bromide are visualized for PCR products using *ppk* primers. **A** represents the strains using Kan as the selectable marker and **B** represents the strains using Tet as the selectable marker. Both gels contain a 1 Kb ladder to represent the DNA fragment size. The WT fragment is 2066 bp, the fragment with *kan* insert is 1558 bp, and the fragment with *tet* insert is 2317 bp. The lanes in figure A are as follows: MG1655 (WT), W3110 (WT), BT2003A (PPK$^-$), CLH403 (PPK$^-$/UvrA$^-$), CLH416 (PPK$^-$/Pol II$^-$), CLH418 (PPK$^-$/Pol IV$^-$), CLH424 (PPK$^-$/RecA$^-$), CLH432 (PPK$^-$/Pol V$^-$), and CLH434 (PPK$^-$/Pol IV$^-$/ Pol V$^-$). The lanes in figure B are as follows: MG1655 (WT), W3110 (WT), CLH408 (DY330 with PPK$^-$), CLH405 (PPK$^-$) , and CLH404 (PPK$^-$/Pol V$^-$). The *ppk* primers used were PPK5BN = cgg gat cca tat ggg tca gga aaa gct ata c and PPK3PH = aaa act gca gaa gct tat tca ggt tgt tcg agt ga.

Strain Construction for *uvrA* Interruptions

The mutated *uvrA* gene was obtained from the parent strain N3055 (λ⁻ IN(*rrnD-rrnE*)1 *uvrA*277::TN10) kindly received from the *E. coli* stock center at Yale University. The *uvr*A277::TN10 DNA was inserted into MG1655 (WT) and BT2003A (PPK⁻) using P1 transduction techniques as described in the methods. Because of difficulty obtaining PCR products that verified the TN10 insert, UV sensitivity was used to screen and verify the strains that were developed. Strains without UvrA are extremely sensitive to UV light so the screen easily displays the strains with and without UvrA. Plates streaked with the selected strains were exposed to 254 nm UV radiation. During the exposure, a screen blocking the UV light was moved across the plate leaving one edge of the plate exposed to UV the entire duration while the opposite edge had no exposure. Figure 6 shows the results of the UV screen for all *uvrA* interrupted strains.

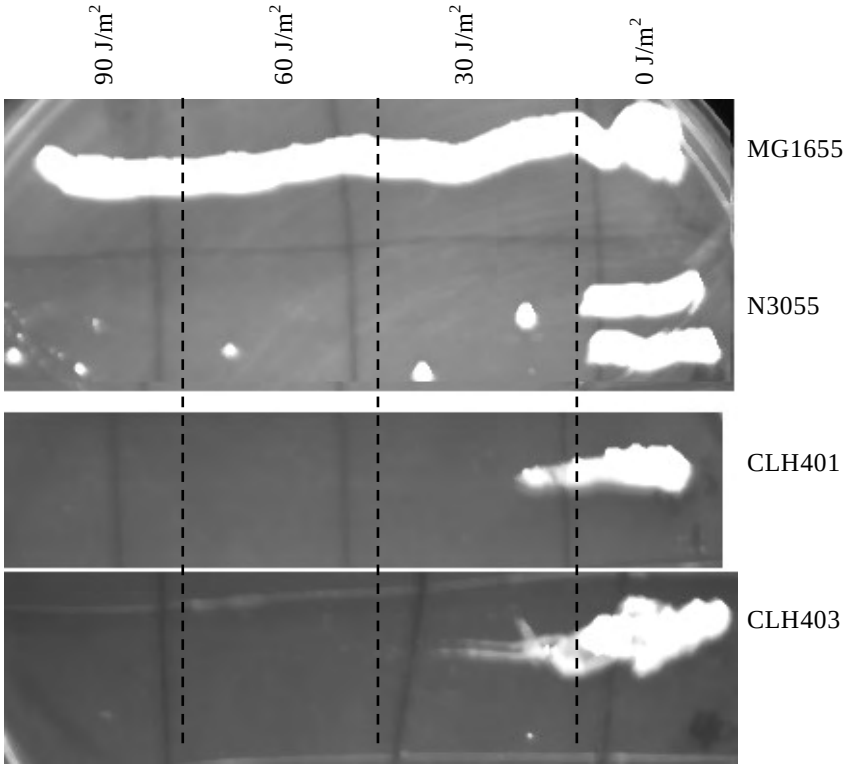


Figure 6 – Has the *uvrA* gene been interrupted in the strains?

Cells streaked on LB agar plates were exposed to 254 nm UV light for a total of 90, 60, 30, and 0 J/m^2. A protective screen covered portions of the plate during the exposure. MG1655 (WT) cells show minimal sensitivity to the radiation. N3055 cells and the two transduced strains (CLH401 (UvrA⁻) and CLH403 (PPK⁻/UvrA⁻)) all have extreme sensitivity to the UV light.

Strain Construction for *recA* Interruptions

The mutated *recA* gene was obtained from the parent strain DY421 (λ^- cI857 Δ(cro-bioA) *recA*::amp) kindly received from the Sawitke laboratory. The *recA*::amp DNA was inserted into MG1655 (WT) and BT2003A (PPK^-) using P1 transduction techniques as described in the methods. To screen and verify the strains that were developed, PCR was used to amplify the genome and compare the size of *recA* fragments. Figure 7 shows the PCR screen for all *recA* interrupted strains.

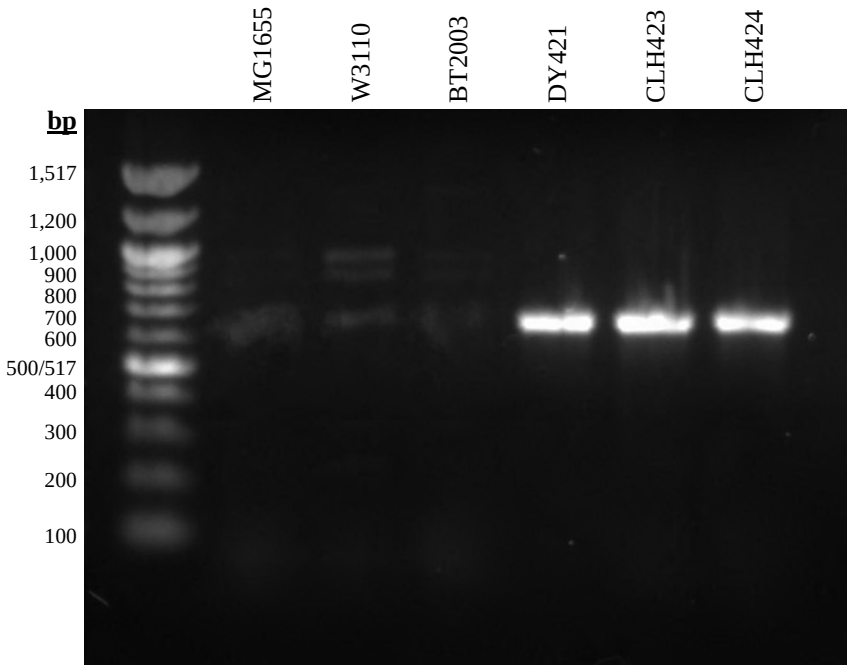


Figure 7 – Has the *recA* gene been interrupted in the strains?

1% agarose gels with ethidium bromide are visualized for PCR products using *recA* primers. The gel contains a 100 bp ladder to represent the DNA fragment size. The 3' primer is inside the *amp* insert so wild type *recA* does not produce any product while the interrupted *recA* produces a PCR product of 663 bp. The lanes in the figure are as follows: MG1655 (WT), W3110 (WT), BT2003A (PPK^-), DY421 (parent $RecA^-$), CLH423 ($RecA^-$), CLH424 ($PPK^-/RecA^-$). The *recA* primers used were RecA 5'= atg gct atc gac gaa aac aa and amp 3' primer = att gct aca ggc atc gtg gtg tca.

Strain Construction for *polB* Interruptions

The mutated *polB* gene was obtained from the parent strain DV08 (*thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 ilv328 thi-1 uvrAA6 Δ(umuDC)596::ermGT ΔpolB::ΩSpc*) kindly received from the Roger Woodgate laboratory. The Δ*polB*::Ω*Spc* DNA was inserted into MG1655 (WT) and BT2003A (PPK⁻) using P1 transduction techniques as described in the methods. To screen and verify the strains that were developed, PCR was used to amplify the genome and compare the size of *polB* fragments. Figure 8 shows the PCR screen for all *polB* interrupted strains.

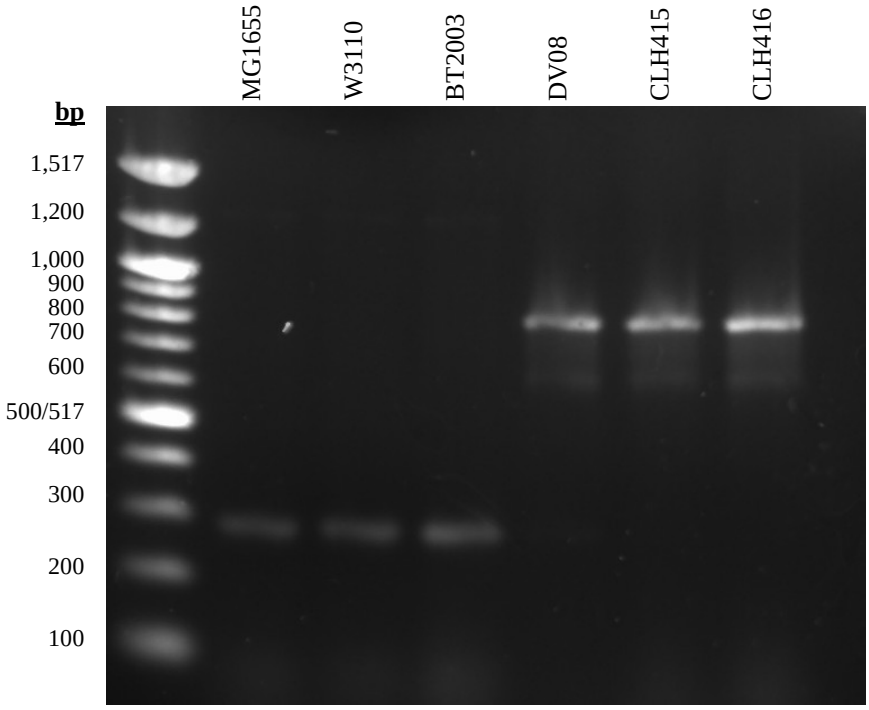


Figure 8 – Has the *polB* gene been interrupted in the strains?

1% agarose gels with ethidium bromide are visualized for PCR products using *polB* primers. The gel contains a 100 bp ladder to represent the DNA fragment size. The WT fragment is 263 bp and the fragment with *spc* insert is 817 bp. The lanes in the figure are as follows: MG1655 (WT), W3110 (WT), BT2003A (PPK⁻), DV08 (parent Pol II⁻), CLH415 (Pol II⁻), CLH416 (PPK⁻/Pol II⁻). The *polB* primers used were 5' polB verification = tct gtc ctg gct ggc gaa cga and 3' polB verification = ccg acg gga tca atc aga aag gtg .

Strain Construction for *dinB* Interruptions

The mutated *dinB* gene was obtained from the parent strain AR30 (*thr-1 araD139 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 sulA211 ΔdinB61::ble*) kindly received from the Roger Woodgate laboratory. The *ΔdinB61::ble* DNA was inserted into MG1655 (WT) and BT2003A (PPK⁻) using P1 transduction techniques as described in the methods. To screen and verify the strains that were developed, PCR was used to amplify the genome and compare the size of *dinB* fragments. Figure 9 shows the PCR screen for all *dinB* interrupted strains.

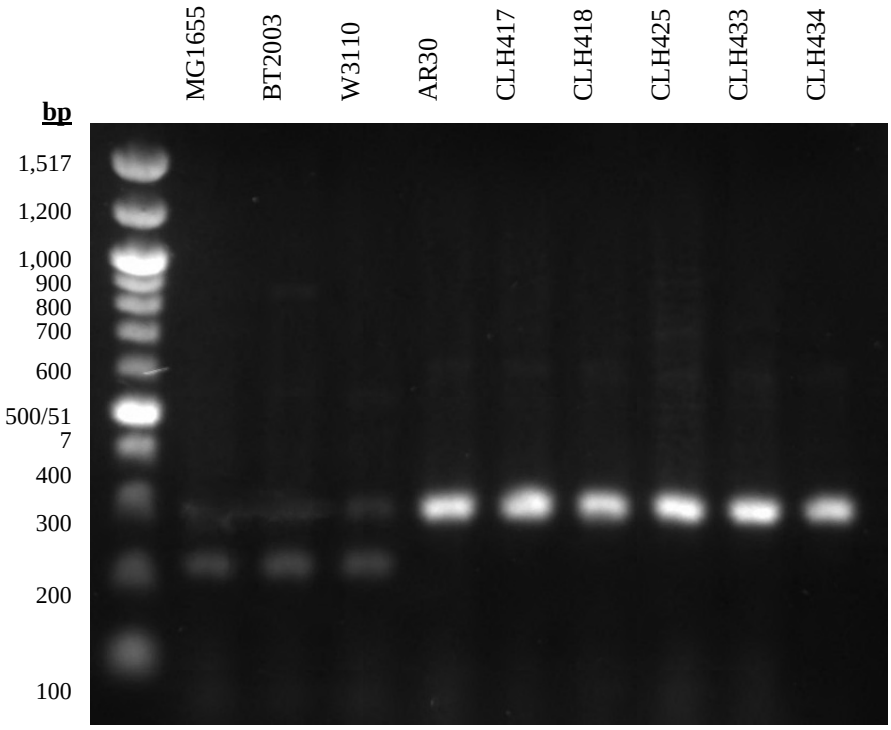


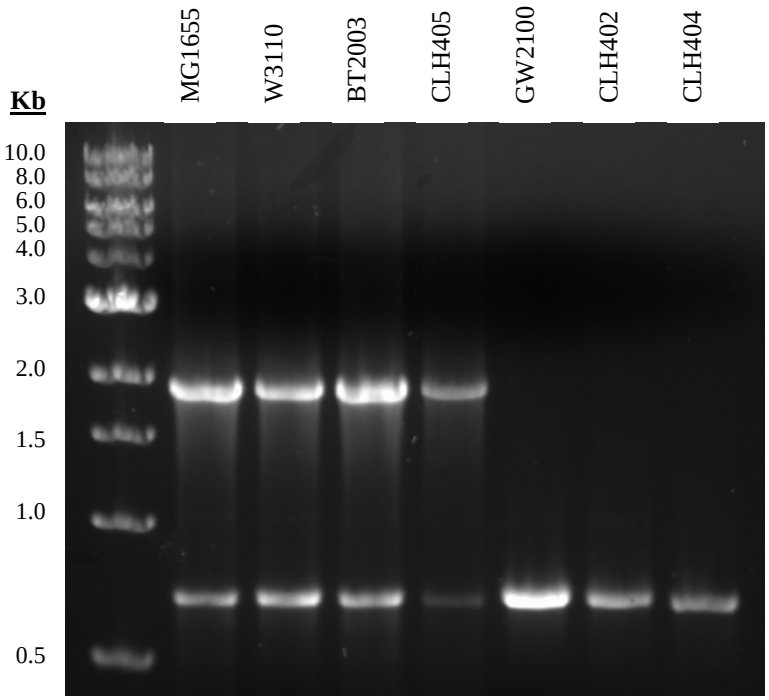
Figure 9 – Has the *dinB* gene been interrupted in the strains?

1% agarose gels with ethidium bromide are visualized for PCR products using *dinB* primers. The gel contains a 100 bp ladder to represent the DNA fragment size. The WT has no strong product, light product at 200 bp, and the Pol IV⁻ fragment containing the *spc* insert is 318 bp. The lanes in the figure are as follows: MG1655 (WT), W3110 (WT), BT2003A (PPK⁻), AR30 (parent Pol IV⁻), CLH417 (Pol IV⁻), CLH418 (PPK⁻/Pol IV⁻), CLH425 (PPK⁻/Pol IV⁻), CLH433 (Pol IV⁻/Pol V⁻), and CLH434 (PPK⁻ /Pol IV⁻/Pol V⁻). The *dinB* primers used were 5' dinB verification = gcc atg acc gag atc ggc gag cag cc and 3' dinB verification = tgt ata ctt tac cag tgt tga gag g.

Strain Construction for *umuDC* Interruptions

The mutated *umuDC* gene was obtained from the parent strain GW2100 (*thr-1 araC14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 qsr' glnV44 galK2 λ⁻ Rac-O hisG4 rfbD1 mgl-51 rpsL3 1kdgK51 xylA5 mtl-1 argE3 thi-1 umuC122::TN5*) kindly received from the *E. coli* stock center at Yale University and DE2302 (*lexA51(*Def) *sulA211 thi-1 Δ(lac-gpt) ilv(Ts) mtl-1 rpsL31 supD43 fadR613::Tn10 purB58 Δ(umuDC)595::cat*) kindly received from the Roger Woodgate laboratory. Initially experiments using Pol V knockouts used *umuC122::TN5*. P1 transduction techniques, as described in the methods, were used to transfer *umuC122::TN5* into MG1655 (WT) and CLH405 (PPK⁻). TN5 contains multiple selectable markers including *str*, *kan*, and *ble*. Because the TN5 contained a *kan* marker, the PPK⁻ strain used for the PPK⁻/Pol V⁻ strain contained the *tet* marker. The multiple selectable markers also caused difficulties when the Pol IV and Pol V double knockout strains needed to be constructed. It was discovered that DE2302 contained a Pol V interruption with *cat* insert allowing the mutated gene to be easily inserted using P1 transduction into MG1655 (WT), BT2003A (PPK⁻), CLH417 (Pol IV⁻) and CLH418 (Pol IV⁻/PPK⁻). To screen and verify the strains that were developed, PCR was used to amplify the genome and compare the size of *umuDC* fragments. Figure 10 shows the PCR screen for all *umuDC* interrupted strains.

A



B

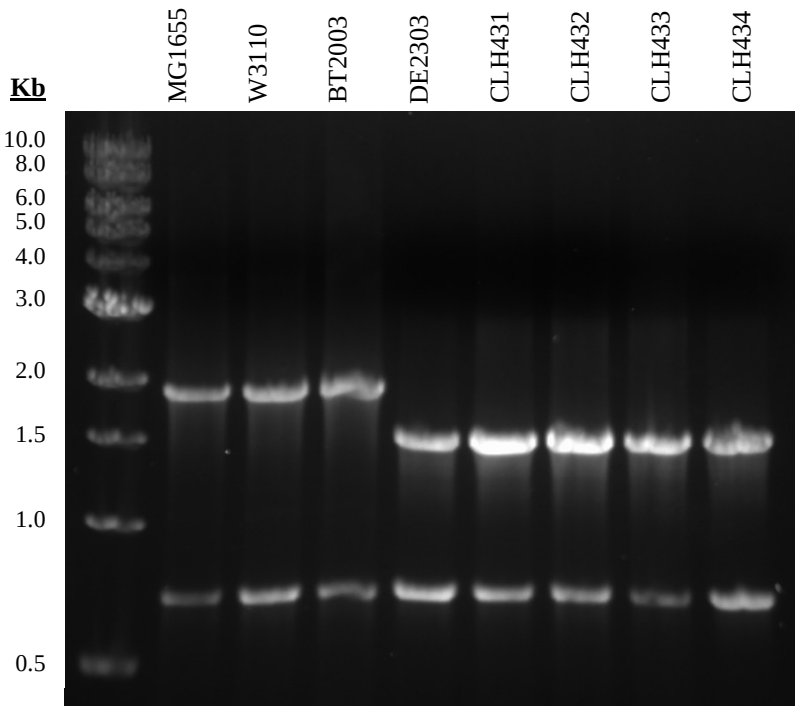
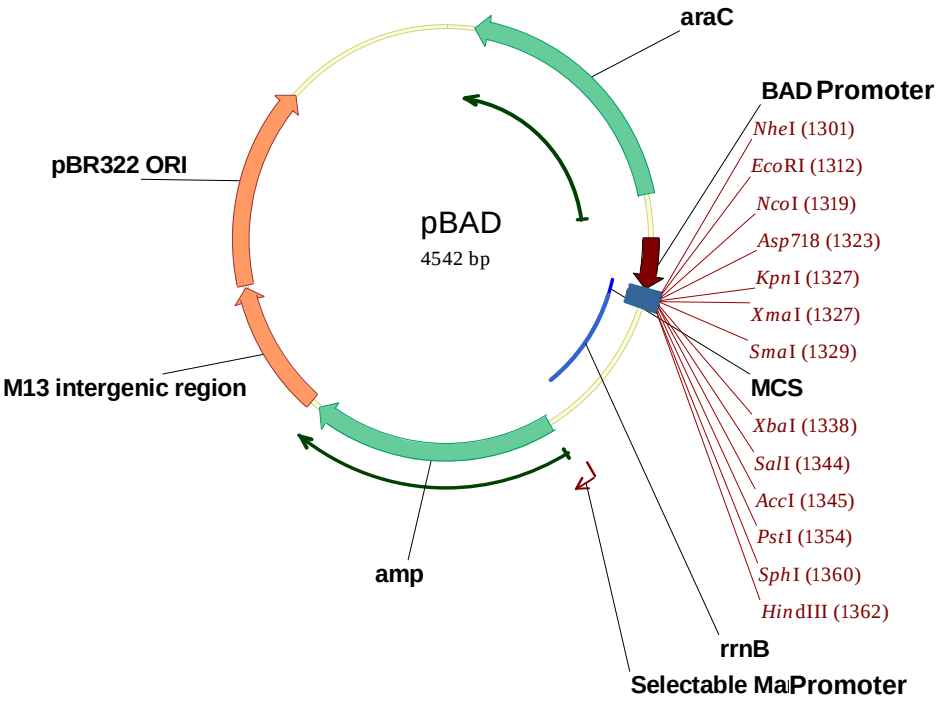


Figure 10 – Has the *umuDC* gene been interrupted in the strains?

1% agarose gels with ethidium bromide are visualized for PCR products using *ppk* primers. **A** represents the strains using *kan* as the selectable marker and **B** represents the strains using *cat* as the selectable marker. Both gels contain a 1 Kb ladder to represent the DNA fragment size. The WT fragment is 2024 bp, the fragment with TN5 insert was too large for a PCR product, and the fragment with *cat* insert is ~1500 bp. All strains contained a non-discretionary band of 75 bp. The lanes in figure A are as follows: MG1655 (WT), W3110 (WT), BT2003A (PPK$^-$), CLH403 (PPK$^-$/UvrA$^-$), CLH416 (PPK$^-$/Pol II$^-$), CLH418 (PPK$^-$/Pol IV$^-$), CLH424 (PPK$^-$/RecA$^-$), CLH432 (PPK$^-$/Pol V$^-$), and CLH434 (PPK$^-$/Pol IV$^-$ /Pol V$^-$). The lanes in figure B are as follows: MG1655 (WT), W3110 (WT), CLH408 (DY330 with PPK$^-$), CLH405 (PPK$^-$), and CLH404 (PPK$^-$/ Pol V$^-$). The *ppk* primers used were 5' umuDC = aaa atc agc agc cta tgc agc gac and 3' umuDC E = aag tgg gtg ccg caa gtg ttt gtc.

Plasmid Design for *ppk* Induction

Insertion of the *ppk* gene into the pBAD24c vector (figure 11) permitted induced overproduction of PPK resulting in high levels of cellular polyphosphates. The designed plasmid, pPPK, was confirmed by DNA sequencing and PCR screening. The arabinose promoter allowed the *ppk* gene to remain off in the presence of glucose and the absence of arabinose. A 35 fold induction of polyphosphates, without any UV radiation, was measured an hour after the promoter was induced with 0.4% arabinose. The plasmid was used to confirm the phenotype of the PPK knockout strain and to confirm that the phenotype of the strain without RpoS was not due to reduced PPK levels.



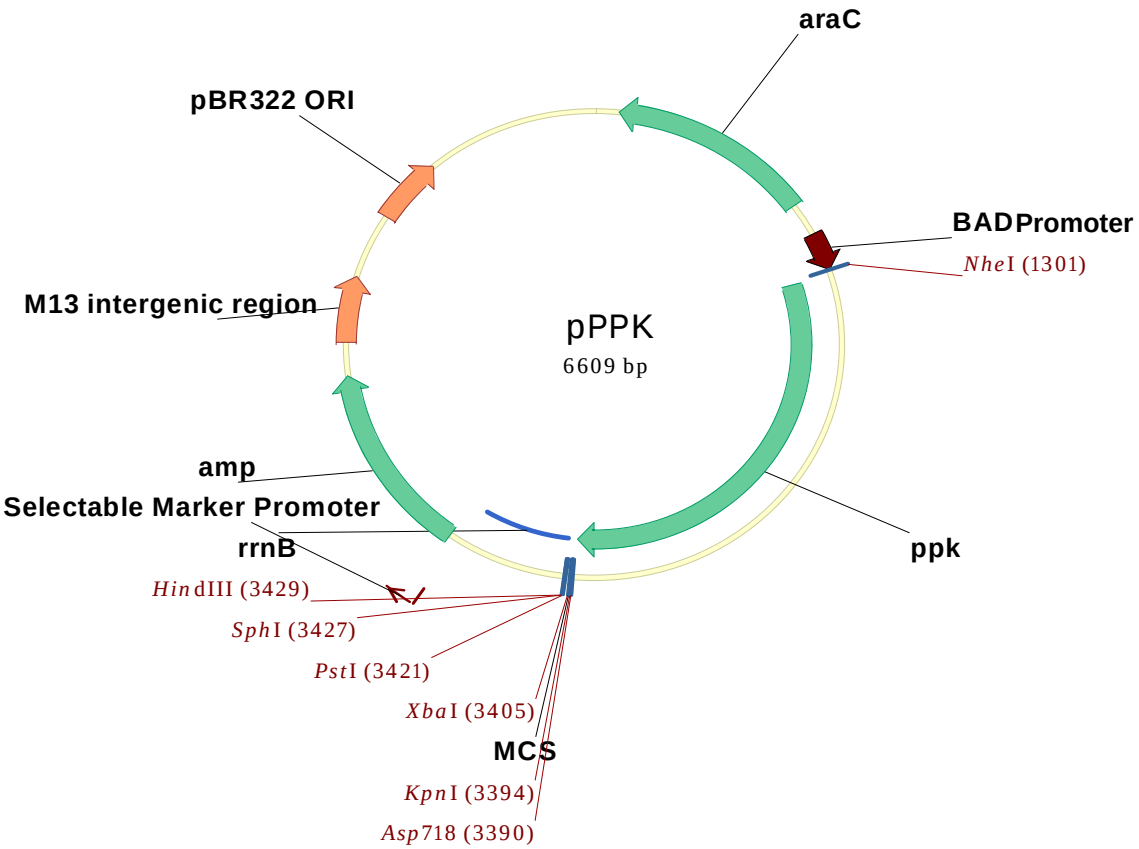


Figure 11 – Construction of pPPK

The pPPK plasmid was created with the pBAD vector and the *ppk* gene. The gene fragment and the vector were digested with NcoI to ligate the gene into the plasmid behind the arabinose promoter. The pPPK plasmid was confirmed by sequencing through the promoter region into the inserted gene region.

Table 1 - Bacterial strains and plasmids

Strain or Plasmid	Relative Genotype	Reference or Source	Text Designation
STRAINS			
MG1655	λ⁻ rph-1 F⁻⁻ *arcA1655 fnr-1655*	Lab Stock	WT
BT2003 A	MG1655 *ppk::kan* (Km^r)	Lab Stock	PPK-
CF5005	MG1655 *rpoS::kan* (Km^r)	Cashel	RpoS-
AR30	*thr-1 araD139 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 sulA211ΔdinB61::ble*	Woodgate	
DV08	*thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 ilv328 thi-1 uvrAA6 Δ(umuDC)596::ermGT ΔpolB::ΩSpc*	Woodgate	
DE2302	*lexA51(*Def) *sulA211 thi-1 Δ(lac-gpt) ilv(Ts) mtl-1 rpsL31 supD43 fadR613::Tn10 purB58 Δ(umuDC)595::cat*	Woodgate	
N3055	λ⁻ *IN(rrnD-rrnE)1 uvrA277::TN10*	*E. coli* SC	
GW2100	*thr-1 araC14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 qsr'⁻ glnV44 galK2* λ⁻ *Rac-O hisG4 rfbD1 mgl-51 rpsL3 1kdgK51 xylA5 mtl-1 argE3 thi-1 umuC122::TN5*	*E. coli* SC	
DY421	λ⁻ cI857 Δ(cro-bioA) recA::amp	Sawitke	
CLH401	MG1655 *uvrA277::TN10*	Lab Stock	UvrA-
CLH402	MG1655 *umuC122::TN5*	Lab Stock	Pol V-
CLH403	BT2003A *uvrA277::TN10*	Lab Stock	UvrA-/PPK-
CLH404	BT2003A *umuC122::TN5*	Lab Stock	Pol V-/PPK-
CLH405	MG1655 *ppk::tet*	Lab Stock	PPK-
CLH408	DY330 *ppk::tet*	Lab Stock	
CLH415	MG1655 *ΔpolB::ΩSpc*	Lab Stock	Pol II-
CLH416	BT2003A *ΔpolB::ΩSpc*	Lab Stock	Pol II-/PPK-
CLH417	MG1655 Δ*dinB61::ble*	Lab Stock	Pol IV-
CLH418	BT2003A Δ*dinB61::ble*	Lab Stock	Pol IV-/PPK-
CLH423	MG1655 *recA::amp*	Lab Stock	RecA-
CLH424	BT2003A *recA::amp*	Lab Stock	RecA-/PPK-
CLH425	CLH417 *ppk::kan* (Km^r)	Lab Stock	Pol IV-/PPK-
CLH431	MG1655 *Δ(umuDC)595::cat*	Lab Stock	Pol V-
CLH432	BT2003A *Δ(umuDC)595::cat*	Lab Stock	Pol V-/PPK-
CLH433	CLH417 *Δ(umuDC)595::cat*	Lab Stock	Pol V-/Pol IV-
CLH434	CLH418 *Δ(umuDC)595::cat*	Lab Stock	Pol V-/Pol IV-/PPK-
PLASMIDS			
pBAD24	*amp* selectable marker	Commercial	pVector
pUC18	*amp* selectable marker	Commercial	
pPPK	pBAD24 derivative carrying *ppk* gene behind the BAD promoter, *amp* selectable marker	Kornberg	pPPK
pBC10	pUC18 derivative carrying *ppk* operon of the *ppk* gene, *amp* selectable marker	Lab Stock (Akiyama *et al.*, 1992)	
pBC29	pUC18 derivative carrying *ppk* gene, *amp* selectable marker	Lab Stock (Akiyama *et al.*, 1993)	

Assay Optimization

Research on polyphosphates has been very limited, as discussed in the introduction. Polyphosphates have been difficult to analyze due to their basic structure and properties, and have been dismissed by many fields of research. Because of the limited work performed on polyphosphates, the tools to study them are also limited and require customization.

The research presented in this thesis is the initial polyphosphate project in the laboratory and at the medial center. The proteins required to perform the *in vitro* studies were purified (PPK1 and scPPX), exclusive reagents required customized preparations (γ-^{32}P polyP), all of the polyphosphate assays were optimized and standard protocols were produced.

How are polyphosphates effectively removed from the cells?

The extraction of polyphosphates from *E. coli* cells was based upon a protocol published in 1998 which utilized polyphosphates poly-ionic properties which are similar to nucleic acid (Ault-Riche *et al.*, 1998). It is required throughout the procedure to perform thorough mixing (vortexing), heating, and pellet resuspensions. Figure 12 depicts the steps of the procedure.

E. coli cultures were pelleted in a 1.5-mL tube and resuspended by adding 0.3 mL of 4 M guanidine isothiocyanate (GITC), 50 mM Tris-HCl, pH 7.0 (GITC lysis buffer). The sample was then either processed directly or frozen in liquid nitrogen and stored at -80°C. The sample was thawed and heated for 5 minutes at 95°C, vortexing vigorously every minute. The samples were then placed in a Branson 2510 bath sonicator for 30 minutes at room temperature. A 25 µl sample was removed for cellular protein concentration. 30 µl of 10% sodium dodecyl sulfate (SDS) was added to the sample and heated to 95°C for 2 minutes. 0.3 mL of 200 proof ethanol was added followed by 8 µl of Glassmilk® from MP Biomedicals (silica slurry). The sample was thoroughly mixed and heated for an additional 30 seconds at 95°C. The tube was immediately microcentrifuged briefly at full speed to pellet the precipitate with DNA, RNA and polyphosphates bound to the silica. The samples were not allowed to sit between heating, spinning, and removing the supernatant to prevent precipitation of SDS. The pellets were then manually resuspended in 0.2 mL of cold wash buffer (5 mM Tris-HCl [pH 7.0], 50 mM NaCl, 5 mM EDTA, 50% ethanol). Because the nucleic acid were still on the silica beads, it was difficult to resuspend the pellet in the wash buffer. Again, the samples were microcentrifuged to gather the pellet. The washing was repeated twice.

To remove the nucleic acid, the washed pellet was resuspended in 200 µl of 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, and 20 µg/mL each of DNaseI and RNaseA and incubated at 37°C for 1 hour. The buffer used in this reaction released the DNA, RNA and polyphosphates from the silica allowing the DNA and RNA to be degraded by the nucleases. After complete digestion, the polyphosphates were rebound to the silica by adding 200 µl wash buffer to the reaction which modified the solution's hydration with the ethanol and increased the salt concentration.

The polyphosphates were collected by microcentrifuging down the silica and a wash was repeated as above with 200 µl wash buffer. After the final spin, the wash buffer was removed and the pellets were left to dry. Polyphosphates were eluted from the pellet by adding 25 µl of

50 mM Tris-HCl (pH 8.0) and heated to 95°C for 1 min. The sample was microcentrifuged and the supernatant containing the polyP was saved. The elution was repeated with an additional 25 µl of 50 mM Tris-HCl (pH 8.0) and the supernatants were pooled for a total of 50 µl of extracted polyP.

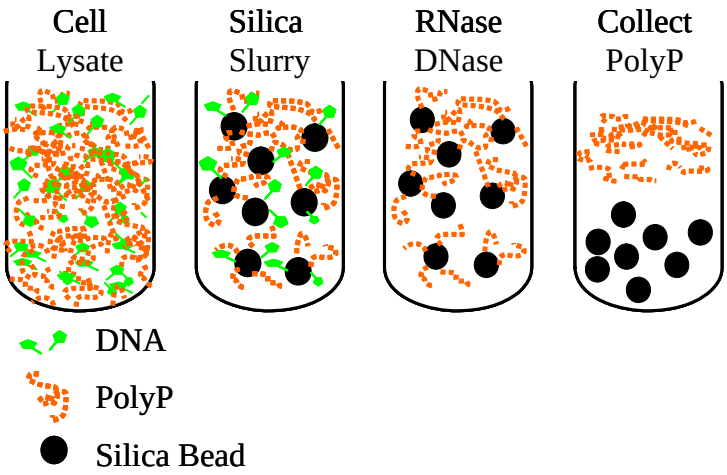


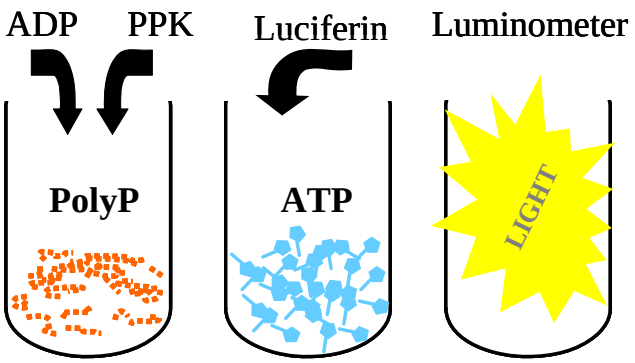
Figure 12 – How are polyphosphates removed from the cells?

To extract polyphosphates from cells, the above four steps are performed. 1) The cells are lysed 2) Nucleic acid and polyphosphates are bound to silica while the remaining cellular particles are removed 3) The nucleic acid is digested 4) Polyphosphates are collected off the silica.

How are polyphosphates concentration measured?

The quantification of the polyphosphates gathered from the *E. coli* cells was based upon a protocol published in 1998 which utilized the reverse reaction of PPK1 to convert the polyphosphates into ATP (Ault-Riche *et al.*, 1998). 10 µL of the extracted polyphosphates and 10 µL of reaction mixture (50 mM Tris-HCl [pH 7.4], 40 mM ammonium sulfate, 4 mM MgCl2, 5 µM ADP, 24,000 U of PPK) were incubated at 37°C for 40 min. The elevated levels of ADP in the reaction mix caused the PPK reaction to degrade the polyphosphates. If the extracted samples contained high levels of polyphosphates, the sample was diluted with water prior to the reaction. After incubation, the reaction mixture was diluted with 80 µL of 100 mM Tris-HCl (pH 8.0), 4 mM EDTA to a total of 0.1 mL. This dilution buffer ensured the reaction was stopped prior to measuring the ATP which was produced. 50 µL of the sample was placed into a well of a black 96 well plate. All samples were added to the wells prior to adding 50 µL of luciferase reaction mixture (ATP Bioluminescence Assay Kit from Roche Diagnostics) to each well. The solutions were mixed by pipeting the solution in and out twice. The time to quantify the light produced by the luciferin and ATP is limited to 20 minutes, so the luciferase reaction mixture was added quickly, and the luminescence was measured immediately. The luminometer measured each well for 10 seconds. ATP concentrations of 10^{-3} to 10^{-12} M were also measured to be used as a standard curve. Concentrations of polyP were calculated in terms of orthophosphate residues. Final cellular concentrations are determined by orthophosphate

residues per mg of protein for the lysed cells. Protein concentrations of the original cell samples were determined utilizing the Bradford method.



$polyP_n + ADP \rightarrow polyP_{n-1} + ATP$

$ATP + D\text{-}Luciferin + O_2 \rightarrow oxyluciferin + PP_i + AMP + CO_2 +$ **Light**

Figure 13 – How are polyphosphates concentration measured?

To measure polyphosphates, the above three steps are performed. 1) Polyphosphate sample is added to PPK and high concentrations of ADP to degrade the polyphosphates into ATP 2) The PPK reaction is stopped and luciferin is added to the resulting ATP 3) The luminescence from the luciferase reaction is measured with a luminometer.

How is the UV exposure kept constant between assays?

Many environmental variables exist while performing the UV exposure assay. Through trial and error many of these variables were found to influence the survival and prevent consistent results. These variables include:

- Volume – A constant volume of cell suspension was placed under the lamp for exposure to have a constant suspension thickness to control the percent of cells shielded from the exposure. Initially, small volumes, 3 ml, were placed in the petri dish under the UV lamp. This volume caused two problems. One, the surface tension of the suspension resulted in varied thickness of the volume. Two, the "bead" of suspension easily moved around the plate during exposure. To control this variable, 20 ml was placed in the petri dish. This was enough volume to cover the entire dish reducing the surface tension, height variance, and suspension movement. 20 ml in a 100 mm petri dish formed in a height of 2.5 mm.
- Temperature – A time lapse exists between the exposure of the first sample and the exposure of the last sample. Cells that had been exposed and left at room temperature while the exposure was completed were able to perform cellular functions such as DNA repair and cell division. This would skew the survival results by increasing the perceived viability. To prevent these situations, all cell samples were placed on ice

throughout UV exposure until they were spread on the LB plates. While under the UV lamp, the petri dish rests on ice to prevent cell death by the lamp heat.

- Light – UV light damages the DNA by forming cyclobutane-pyrimidine dimers. One cellular function to repair this damage also uses light but with wavelengths of 320 to 500 nm. DNA photolyases are highly efficient light-driven DNA repair enzymes which revert the genome damaging effects caused by UV. Their catalytic mechanism employs the light-driven injection of an electron onto the DNA lesion to trigger the cleavage of cyclobutane-pyrimidine dimers (Essen and Klar, 2006). The survival assay was performed both in complete darkness and under the fluorescent lights of the laboratory. Significant differences in survival were not detected, but to remove this variable the assay was performed without direct light. There was not complete darkness because too many errors and contaminations occurred under these circumstances.

- Sample concentration – The 20 ml of constant sample volume prevented variations in cell shielding due to height differences of the sample. Cell shielding variances also occur if the sample concentration was not consistent between assays. Higher concentrations of cells resulted more cell shielding. To overcome this problem, a constant 2.5×10^8 cells/ml concentration was used for each sample even if the initial culture concentrations were not the same.

- Culture growth phase – The growth status of the cells caused variations in the survival results. The qualitative trend of the results remained consistent but the quantitative data did not. Unless otherwise mentioned, the experiments were performed with cells at stationary phase.

- Culture media – The culture media affected the physiology of the cells. Rich media resulted in shorter doubling time. Similar to the growth phase variations, the qualitative trend of the results remained consistent but the quantitative data did not. Unless otherwise mentioned, the experiments were performed with cells grown in MinA media. This media was selected because it does not contain Cl^- which interacted with the cisplatin. Also, the composition of MinA is more defined, containing fewer variables, than LB.

- UV lamp height – The intensity of the 254 nm UV light reduced as the height of the lamp from the sample increased. To ensure constant rate of exposure, the UV was measured with a Spectroline® Digital Radiometer model DRC-100X throughout the experiment. The sample was placed on packed ice while being exposed. During the experiment, the ice melted, and the height of the sample lowers. Therefore between each sample, the radiometer measured the UV intensity to ensure each sample received the same quantity.

- Exposure media – Previously in the laboratory it was observed that exposing the cells to UV while in LB media gave different results than when the cells were suspended in 100 mM $MgSO_4$. The compilation of substances in the LB could cause effects on the exposure. There was no difference between exposure in 100 mM $MgSO_4$ or in MinA media. Yet, to remain constant, the samples were suspended in 100 mM $MgSO_4$ during each exposure.

A precise procedure was followed for the UV survival assays to try to eliminate as many of these variables. Even under this control, small survival fluctuations were seen between experiments for the same strain.

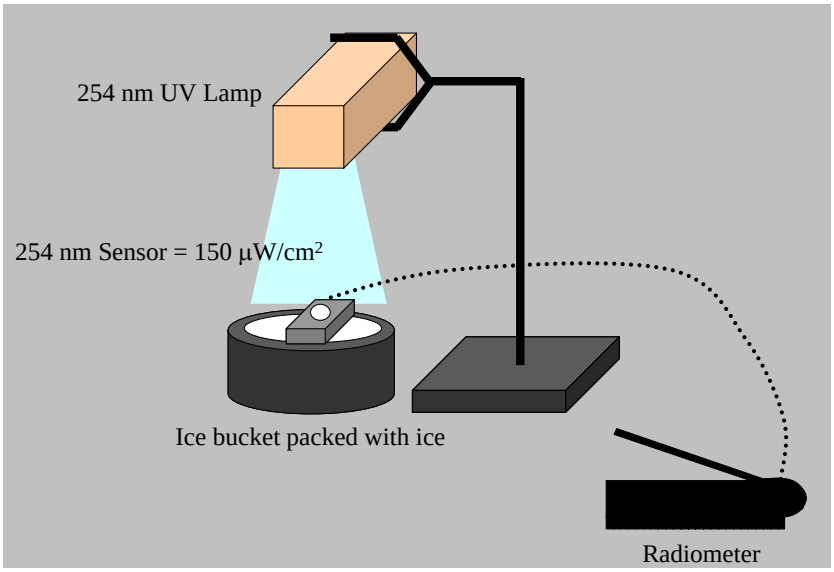
A single colony of cells were grown overnight in 5 ml of LB rotating at 37 °C. This was the inoculating culture. Cells from the inoculating culture were added to three separate flasks containing MinA media for a final cellular concentration of 1.0×10^5 cells/ml. The cultures were grown in liquid MinA media shaking at 37 °C to stationary phase to a final cellular concentration of 1.8 to 2.0×10^9 cells/ml. The concentration of the cultures was determined using the optical density at 600nm wavelength.

Exactly 5.0×10^9 cells in a constant 20 ml of 100 mM $MgSO_4$ media in a 100 mm petri dish were exposed to 254 nm UVC light by a model UVGL-25 Mineralight® lamp by UVP, Inc. To ensure consistent exposure, the UV was measured with a Spectroline® Digital Radiometer model DRC-100X. One ml samples were gathered at designated UV quantities. The samples were diluted in LB, and spread on LB plates before incubating overnight at 37 °C. The radiation and incubation were performed without direct light to prevent DNA repair by photolysases (Essen and Klar, 2006). All samples remained on ice from the time the concentration of the culture was determined until the cells were diluted in 10-fold increments and spread on LB plates.

Colony forming units (CFU) were counted on the LB plates. The samples were diluted prior to being spread on the plates at a concentration which resulted in 50-200 CFU per plate. Colony counts in this range allowed accurate representation of the survived cells and accurate counting of the colonies on the plate. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at a specified quantity of UV.

Some variables, such as culture media and growth phase, were modified for certain experiments. The above procedure was adjusted accordingly.

A



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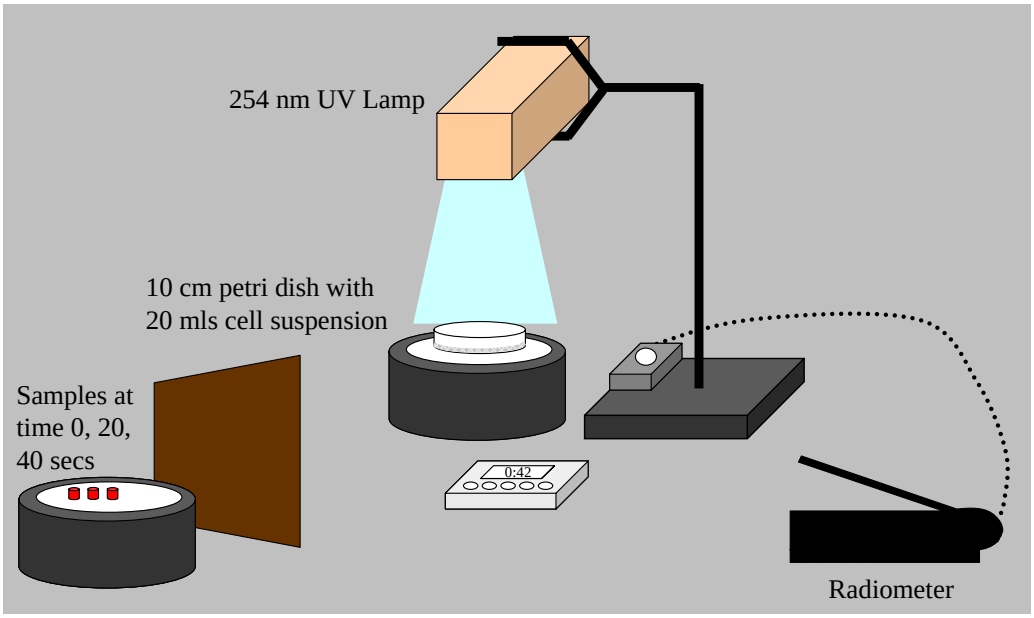


Figure 14 – How is the UV exposure kept consistent between assays?

The above diagrams depict the setup of the apparatus during the UV survival assay. **A** shows how the equipment is prepared and **B** shows the setup during exposure.

Cell Survival Following DNA Damage

Survival Following UV Radiation

The presence of polyphosphates in a cell increases the cellular viability following environmental stresses that trigger the general stress response, controlled by RpoS. DNA damage caused by UV radiation is an additional environmental stress which triggers DNA repair mechanisms including, the SOS response but not the general stress response. To determine if polyphosphates participate in cellular viability following UV radiation, survival assays were performed.

Do polyphosphates increase cell viability after UV radiation?

Wild type *E. coli* cells and cells with an interrupted *ppk* gene (PPK^{-} cells) were grown, treated and radiated by UV light. Under all conditions, PPK^{-} cells had decreased survival for exposures ranging from 0 to 150 J/m^2 (Figure 15). During preliminary experiments, it was observed that variances in initial conditions, such as cell density, led to significant fluctuations in the results. While each experiment showed increased sensitivity for the PPK^{-} cells, the degree of survival after exposure to 150 J/m^2 ranged from a 10 fold to 100 fold difference relative to wild type cells, depending on initial culture conditions. Therefore, the radiation procedure was performed consistently to ensure the same exposures across all of the samples and experiments.

Wild type and PPK^{-} cells were grown overnight to stationary phase in MinA media. Three separated flasks of cultures were grown for each strain per experiment. The stationary phase cultures were diluted to 5.0×10^9 cells in a constant 20 ml of 100 mM $MgSO_4$ and were exposed to 254 nm UVC light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were diluted in LB and spread on LB plates before incubating overnight at 37 °C. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 150 J/m^2 for 9 cultures and over 3 separate days of experiments.

As depicted in Figure 15, the PPK^{-} strain had a 5 fold decrease in viability at 150 J/m^2 of UV radiation compared to wild type with a p value of <0.005. These results indicate *E. coli* cells lacking PPK have decreased survival after exposure to UV radiation.

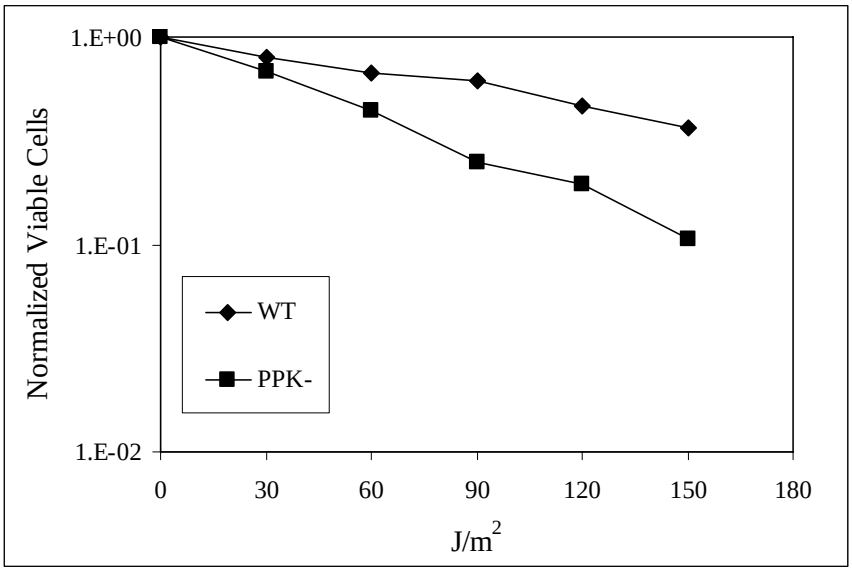


Figure 15 – Do Polyphosphates Increase Cell Viability After UV Radiation?

WT and PPK$^-$ cells are designated with ◆ and ■, respectively. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and exposed to 254 nm UV light. The viability was determined by plating the radiated samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed 9 times in triplicate. The experiment was proved statistically significant using the Student t-test comparing WT and PPK$^-$ cells at 150 J/m^2. $p < 0.0005$

Can the increased sensitivity in cells lacking polyphosphates be reversed?

Using a strain constructed with an interrupted *ppk* gene, it was shown above that cells lacking PPK have decreased survival after exposure to UV radiation. But, to ensure the only mutation in the PPK$^-$ strain, which caused the decreased survival, was the *ppk* interruption, PPK$^-$ must be rescued with reintroducing *ppk*. Therefore, to confirm the increased sensitivity resulted only from the lack of PPK, the PPK$^-$ cells were transformed with a plasmid (pPPK) carrying the *ppk* gene behind an inducible BAD promoter (Guzman et al., 1995).

Similar to the standard experimental conditions described above, overnight stationary triplicate cultures of WT, PPK$^-$, PPK$^-$/pPPK, or PPK$^-$/pBAD cells were grown in MinA media containing succinate instead of glucose as the carbon source. One hour prior to UV radiation, the plasmids were induced by introducing 0.04% arabinose to the cultures. After continuing the incubation for one hour, the cultures were diluted to exactly 5.0 x 10^9 cells in a constant 20 ml of 100 mM $MgSO_4$ and were exposed to 254 nm UVC light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were diluted in LB and spread on LB plates before incubating overnight at 37 °C. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 150 J/m^2 for 4 separate cultures and over 2 separate days of experiments.

The PPK⁻/pPPK cells were rescued and behaved like WT cells with a not significant p value, indicating no difference between PPK⁻/pPPK and WT. Cells transformed with the vector alone (PPK⁻/pBAD) showed no phenotypic survival difference compared to PPK⁻ (Figure 16). Statistical analysis of PPK⁻/pPPK versus PPK⁻/pBAD showed a significant difference like WT versus PPK⁻ with a p value of <0.005.

The difference in the survival phenotype between WT and PPK⁻ was due only to the mutation of the *ppk* gene and no other unplanned modification in PPK⁻.

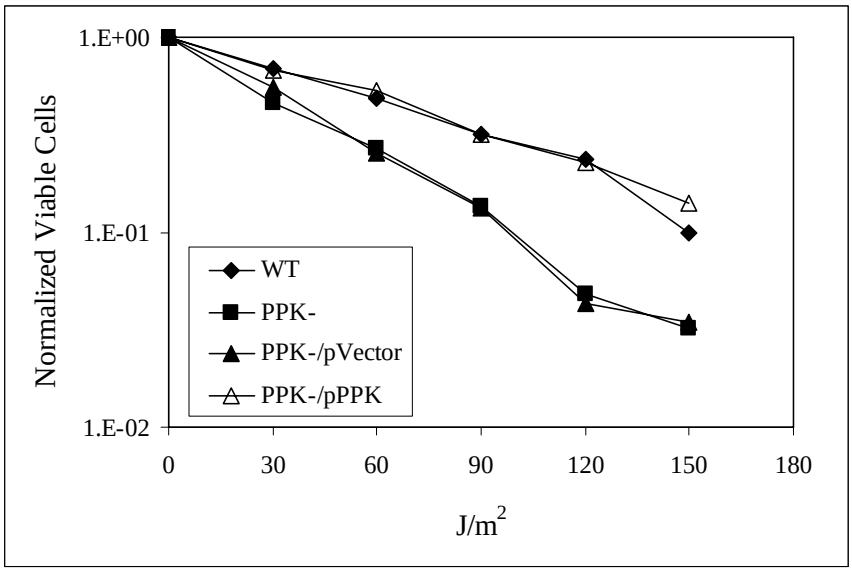


Figure 16 – Can the increased sensitivity in cells lacking polyphosphates be reversed?

WT and PPK⁻ cells are designated with ◆ and ■, respectively. PPK⁻ cells containing plasmid with and without *ppk* gene are designated by ▲ and △, respectively. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and exposed to 254 nm UV light. The viability was determined by plating the radiated samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed 4 times in triplicate. The experiment was proved statistically significant using the Student t-test comparing WT and PPK⁻/pPPK, WT and PPK⁻/pVector, PPK⁻ and PPK⁻/pPPK, PPK⁻ and PPK⁻/pVector, at 150 J/m^2. p values are NS, <0.005, <0.005, and NS, respectively.

Do the rescued cells have elevated polyphosphate levels?

The PPK⁻ cells were able to be rescued by introducing a plasmid caring the *ppk* gene, but the addition of the *ppk* gene does not ensure the polyphosphate levels were increased back to WT levels. To confirm that this plasmid produced the PPK protein and elevated the polyphosphate quantity in the cells, the polyphosphate levels were measured.

Similar to the standard culture growth conditions described above, overnight stationary triplicate cultures of WT, PPK⁻, PPK⁻/pPPK, or PPK⁻/pBAD cells were grown in MinA media containing succinate instead of glucose as the carbon source. One hour prior to harvesting the

cells, the plasmids were induced by introducing 0.04% arabinose to the cultures. After incubating, 1.0 x 10^9 cells were centrifuged into a pellet, resuspended in 300 μL 4M GITC lysis buffer, and frozen in liquid nitrogen. The samples were stored at -80 °C prior to executing the polyphosphate extraction procedure and the polyphosphate measurement procedure. (Methods)

Measurement of polyphosphate levels showed a 500 fold increase in polyphosphate concentration in PPK⁻/pPPK cells, verifying the overproduction of PPK and heightened synthesis of cellular polyphosphates. The level of polyphosphates remained unchanged in PPK⁻ cells transformed with pBAD (Table 2).

The presence of pPPK in the PPK⁻ cells elevated the level of polyphosphates far beyond the WT level. WT polyphosphate levels were 7 times higher that PPK⁻. PPK⁻/pPPK cells had 56 fold increase over PPK⁻ and 8 fold increase over WT cells. The viability of the strain returned to that of WT, so the excess polyphosphates did not increase the viability above the level of WT. This suggests polyphosphates are not a limiting factor in a survival mechanism and excess polyphosphates do not improve the cells ability to survive.

Table 2 – PolyP concentrations after one hour of induction (pmol/mg protein)

STRAIN	INDUCED PLASMIDS
Wild type	1,400
PPK⁻	200
PPK⁻/pPPK	11,200
PPK⁻/pBAD	50

Is the decreased survival in cells lacking polyphosphates growth phase specific?

The survival results of WT and PPK⁻ shown above were grown in minimal media into stationary phase growth. Since polyphosphates have been reported to affect the stress response mechanism, the difference in survival seen after UV radiation may be caused by the stress response activated by the stationary growth conditions. To ensure polyphosphates have a direct effect on cell survival following DNA damage and not due to an indirect effect through the stress response, the growth conditions were varied between log and stationary for cells radiated with UV.

Following similar standard culture growth conditions described above, three separate culture flasks of WT and PPK⁻ cells were grown in liquid MinA media shaking at 37 °C to either stationary phase or log phase with final cellular concentrations of 1.8 to 2.0 x 10^9 cells/ml or 0.4 x 10^9 cells/ml, respectively. 5.0 x 10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light. Samples were gathered at designated UV quantities. The samples were diluted in LB and spread on LB plates before incubating overnight at 37 °C. CFU's were counted on the LB plates. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 150 J/m^2 for the 3 separate cultures in the experiment.

Whether in exponential or stationary growth phase, the wild type cells have increased viability compared to the PPK⁻⁻ cells following UV exposure (150 J/m^2) (Figure 17). This data suggests that the increased survival observed with DNA damage is not limited to polyphosphate's involvement in the general stress response that occurs as cells enter the quiescent state. If the difference in survival was only seen in stationary phase, it could be argued that polyphosphates are not involved in the SOS DNA repair response, and that polyphosphate's involvement in the general stress response was the sole cause of the difference. However, based upon the data (Figure 17), polyphosphates are involved with the SOS DNA repair response to increase cell survival after UV DNA damage regardless of growth phase.

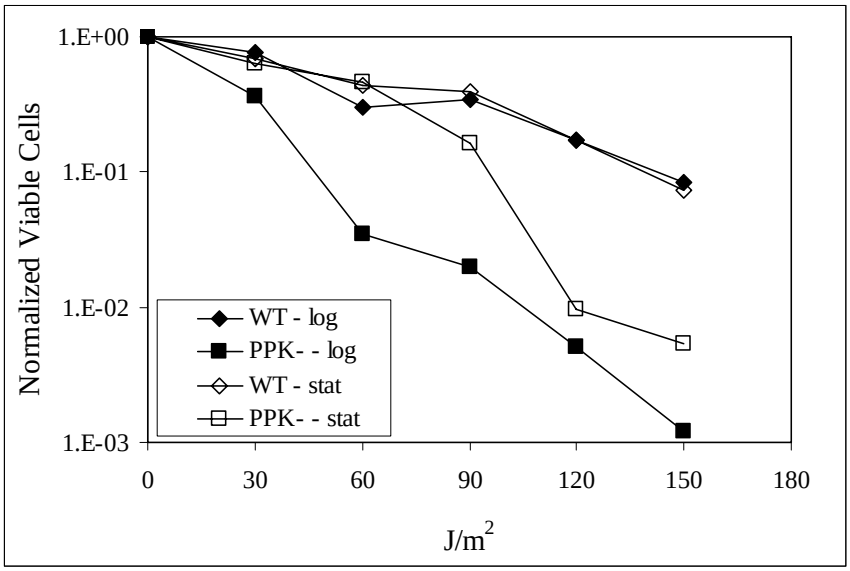


Figure 17 – Is the decreased survival in cells lacking polyphosphates growth phase specific?

WT and PPK$^-$ cells in stationary phase are designated with ◆ and ■, respectively. WT and PPK$^-$ cells in log phase are designated with ◇ and □, respectively. Cells are grown in MinA minimal media to either log or stationary growth phase at 37 °C and exposed to 254 nm UV light. The viability was determined by plating the radiated samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed 3 times in triplicate. The experiment was proved statistically significant using the Student t-test comparing WT and PPK$^-$ in log growth and WT and PPK$^-$ in stationary growth. p values are < 0.0005 and < 0.005, respectively.

Is the decreased survival in cells lacking polyphosphates growth rate specific?

The proceeding section proved that the increased sensitivity to UV DNA damage in the PPK⁻⁻ cells is not dependent on the growth phase, but all of the cultures were grown in minimal media which results in a slower growth rate. To determine if varying viability is due to the growth rate, WT and PPK⁻⁻ cultures were grown in LB rich media to log phase growth prior to performing the UV survival assay.

Following similar standard culture growth conditions described above, three separate culture flasks of WT and PPK⁻ cells were grown in the liquid LB media shaking at 37 °C to log phase with final cellular concentrations of 0.4 x 10^9 cells/ml. 5.0 x 10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light. Samples were gathered at designated UV quantities. The samples were diluted in LB and spread on LB plates before incubating overnight at 37 °C. CFU's were counted. The data were normalized to 0 J/m^2 UV light.

The survival phenotype difference between wild type and PPK⁻ cells are not dependent on the culture growth rate. PPK⁻ cells are sensitive to UV radiation during slow exponential growth when cultured in a minimal medium (Figure 17). But the effect of polyphosphates on survival is not limited to slow growth. PPK⁻ cells rapidly growing in a rich medium are highly sensitive to UV (Figure 18). The overall survival of both wild type and PPK⁻ cells are more sensitive when growing with a short (21 min) versus a long (90 min) doubling time. The doubling time of rapidly growing cells was shorter than the approximate 45 minute needed to elicit the Pol V and Pol IV SOS response (Pham *et al.*, 2001), hence augmenting genomic instability, caused by the DNA damage and partially replicated DNA, can occur. This agrees with previous studies that showed the difference in cell survival based solely on the growth rate (Morton and Haynes, 1969; Stapleton and Engel, 1960; Stapleton, 1955).

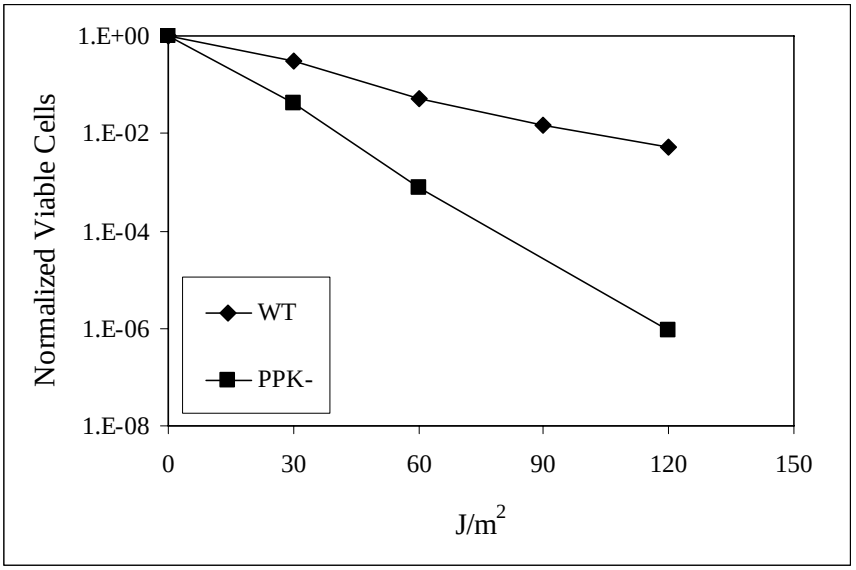


Figure 18 – Is the decreased survival in cells lacking polyphosphates growth rate specific?

WT and PPK⁻ cells are designated with ◆ and ■, respectively. Cells are grown in rapid growth LB medium at 37 °C and radiated with UV while in log growth phase with a 21 minute doubling time. The viability was determined by plating the radiated samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation.

Survival Following Other DNA Damage Agents

Is the decreased survival in cells lacking polyP specific to DNA adducts caused by UV?

All of the above results used UV radiation as the DNA damaging agent to trigger the SOS response; yet, the SOS response can be triggered by other forms of DNA damage. To verify that

the difference in viability between WT and PPK⁻ cells was not specific to pyrimidine dimer adducts formed by UV radiation, cisplatin was used to cause DNA damage and trigger the SOS response. The chemotherapeutic drug cisplatin forms DNA adducts through intra or inter-strand crosslinks, thus causing DNA damage and inhibiting cellular processes, including replication and transcription (Wozniak and Blasiak, 2002).

To evaluate if the decreased viability was limited to UV DNA damage, WT and PPK⁻ cells were cultured over night in liquid MinA media shaking at 37°C to stationary phase to a final cellular concentration of 1.8 to 2.0 x 10^9 cells/ml. 2.5 x 10^8 cells were incubated 1 hour at 37°C in 1 ml of MinA media that contained varying concentrations (0 to 60 μg/ml) of cisplatin. The samples were diluted in LB and spread on LB plates before incubating overnight at 37°C. CFU's were counted on the LB plates. The data were normalized to 0 μg/ml of cisplatin, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 60 μg/ml of cisplatin for the 3 separate cultures in the experiment.

The PPK⁻ cells had increased sensitivity to cisplatin, similar to that seen with UV exposure (Figure 19). There is nearly a 100-fold difference in survival when the cells were exposed to 60 μg/ml cisplatin. Even though cisplatin forms a different type of DNA lesion, it also triggers the NER mechanism and the SOS response, like UV radiation. As expected, polyphosphate deficient cells behave in a similar manner for both DNA damaging agents.

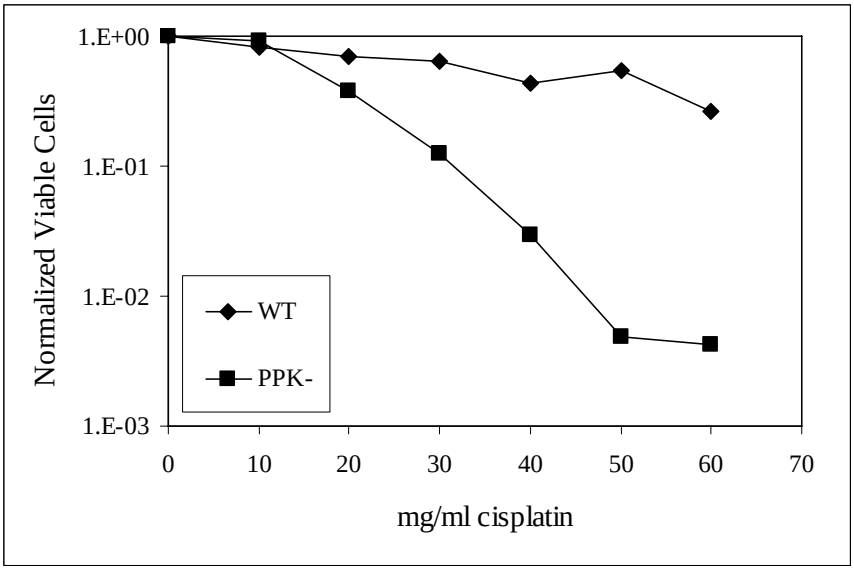


Figure 19 – Is the decreased survival in cells lacking polyP specific to DNA adducts caused by UV?

WT and PPK⁻ cells are designated with ◆ and ■, respectively. Cells were grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and incubated in varying concentrations of cisplatin for 1 hour at 37 °C. The viability was determined by plating the samples on LB agar. Percent survival is determined as the viable cell number at each cisplatin concentration divided by the viable cell number without cisplatin. The experiment was performed 3. The experiment was proved statistically significant using the Student t-test comparing WT and PPK⁻ cells at 60 μg/ml cisplatin. $p = < 0.005$

Is the decreased survival in cells lacking polyphosphates specific to single stranded DNA damage?

Both UV and cisplatin DNA damage form adducts that cause a bend in the DNA helix. The repair mechanisms for both forms of DNA are therefore similar, most commonly NER. To test if the variance in survival between WT and PPK⁻ cells was due to a mechanism specific to DNA adducts, the survival assay was performed using a DNA damaging agent which causes double stranded breaks. Gamma irradiation produces double stranded breaks in cellular DNA, thus activating recombination for DNA repair, a repair mechanism different than those for DNA lesions.

WT and PPK⁻ cells were cultured over night in liquid MinA media shaking at 37 °C to stationary phase to a final cellular concentration of 1.8 to 2.0 x 10^9 cells/ml. Exactly 5.0 x 10^9 cells in a constant 20 ml of 100 mM $MgSO_4$ was placed in a 100 mm petri dish. The dish was placed in a cesium irradiator at room temperature. The exposure was 2.99 Gy/min and 0.5 mL samples were withdrawn from exposed sample and diluted in LB media. The samples were spread on LB plates before incubating overnight at 37 °C. CFU were counted. The data were normalized to 0 Gy, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 400 Gy for the 3 separate cultures in the experiment.

The PPK⁻ cells had decreased viability compared to WT cells (Figure 20). A difference of over ten fold in survival appears when the two cell types were exposed to 300 or 400 Gy, suggesting that the role of polyphosphates in cell survival after DNA damage is not limited to a single, specific DNA repair mechanism in the SOS pathway. Instead, it appears to have a more universal role in the SOS response pathway.

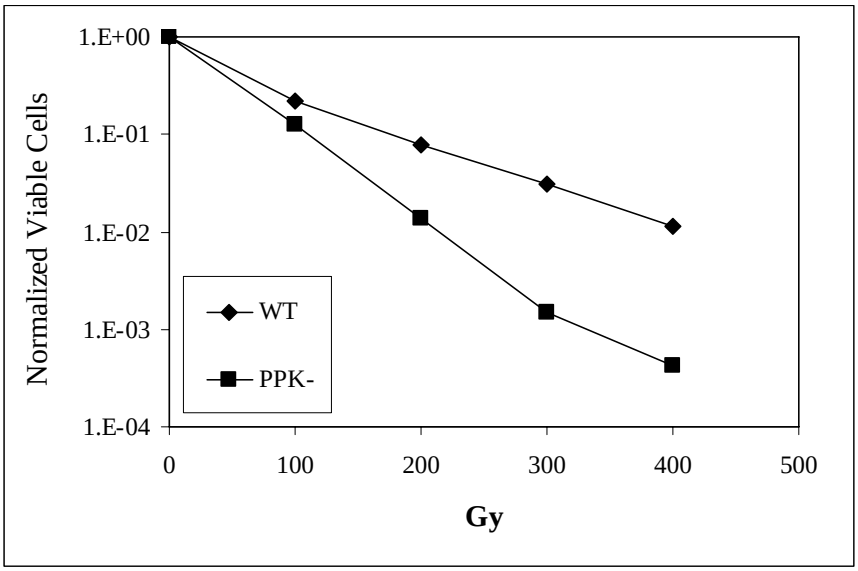


Figure 20 – Is the decreased survival in cells lacking polyphosphates specific to single stranded DNA damage?

WT and PPK$^-$ cells are designated with ◆ and ■, respectively. Cells were grown at 37 °C in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C. 5 x 10^9 cells were suspended in 20 ml of MinA and placed into a Petri dish before exposed to gamma irradiation. The viability was determined by plating the samples on LB agar. Percent survival is determined as the viable cell number at each gamma irradiation quantity divided by the viable cell number without irradiation. The experiment was performed in triplicate. The experiment was proved statistically significant using the Student t-test comparing WT and PPK$^-$ cells at 400 Gy. $p = < 0.005$

Polyphosphate Dynamics

What happens to polyphosphate levels following DNA damage?

Polyphosphates appear to assist in cell survival following DNA damage at least partially independent of growth rate, growth phase, and DNA damage source. Elevations in polyphosphate levels have been observed following exposure to other non-DNA damaging environmental challenges that activate the general stress response pathway (Ault-Riche et al., 1998), but the polyphosphate levels following DNA damaging stress is unknown. To examine dynamics in polyphosphates following DNA damage, polyphosphates were measured in WT and PPK$^-$ cells following DNA damage.

Similar to the standard experimental conditions described above, overnight stationary triplicate cultures of WT WT and PPK$^-$ cells were grown in MinA media. The stationary cultures were diluted to exactly 5.0 x 10^9 cells in a constant 20 ml of 100 mM $MgSO_4$ and were exposed to 120 J/m^2 of 254 nm UVC light in a 100 mm petri dish. The cultures were radiated according to the procedure in the Methods. After radiation, the cells were concentrated to the cell density of stationary phase, and allowed to grow shaking at 37°C. Samples were collected at time points following radiation, starting with 30 minutes and ending with 5 hours. The samples were resuspended in 300 μL GITC lysis buffer and frozen in liquid nitrogen. The samples were stored

at -80°C prior to executing the polyphosphate extraction procedure and the polyphosphate measurement procedure. (See Methods)

After lysing the cells, polyphosphates were extracted by binding DNA, RNA, and polyphosphates to silica, degrading the DNA and RNA, and eluting the polyphosphates from the silica. The polyphosphates were then converted to ATP using the PPK reverse reaction, and the ATP was reacted with luciferin to produce light for measurement. The final cellular concentrations were determined in moles of orthophosphate residues per mg of cellular protein. Protein concentrations of the original cell samples were determined utilizing the Bradford method. The assays were performed in triplicate, and the values averaged.

After exposure to UV, there was a transient increase in polyphosphate concentration in wild type cells (Figure 21). The levels of polyphosphates increased approximately three-fold in stationary phase cells following their exposure to UV. Polyphosphate levels began to increase within 30 min after radiation and returned to normal levels 2 hours later (Figure 21). This increase in polyphosphate concentration occurred in the same time frame as the SOS mediated induction of error-prone polymerases Pol IV and Pol V. The PPK^{-} cells did not show an increase in polyphosphates at any time after the UV exposure.

As with other environmental stresses, DNA damage triggers an increase of polyphosphates. It is not known if this increase was due to increased transcription of *ppk* and production of PPK, increased production of polyphosphates by elevated PPK activity, or decreased degradation of polyphosphates by PPX.

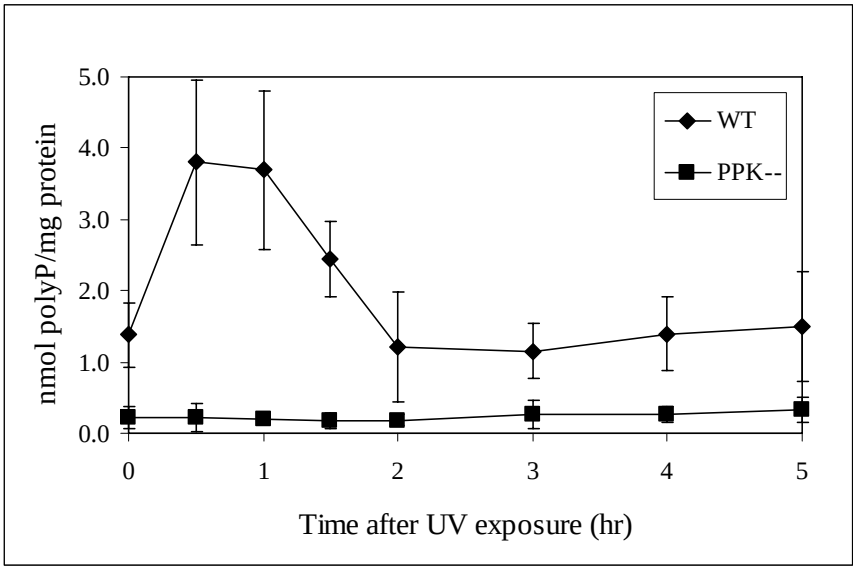


Figure 21 – What happens to polyphosphate levels following DNA damage?

WT and PPK^{-} cells are designated with ◆ and ■, respectively. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C. 5 x 10^9 cells were suspended in 20 ml of MinA and placed into a Petri dish before exposed to 254 nm 150 J/m^2 UV radiation. The cells were concentrated back to their original overnight concentration and incubated at 37 °C without light for designated time (0-5 hours). The polyphosphates were extracted and measured from the cells. The experiment was performed in triplicate. The average values were determined along with the standard deviation.

Polyphosphates, DNA Damage and RpoS

Are cells without RpoS sensitive to DNA damage?

The growth rate and growth phase were varied, compared to the standard procedures, to investigate if polyphosphates involvement in the general stress response was related to the decreased survival after DNA damage. As mentioned in the introduction, $RpoS^-$ is the sigma transcription factor which activates transcription of genes needed for survival by the general stress response. It's been reported that polyphosphate levels did not increase in strains without RpoS (Ault-Riche et al., 1998). Based on this information, the cell survival following DNA damage was evaluated for a strain without *rpoS* to investigate if RpoS had any influence on the survival of cells after triggering the SOS response.

A strain with the *rpoS* gene interrupted was exposed to UV radiation, and the survival phenotype was compared to those of wild-type and PPK^{--} cells. WT, PPK^-, and $RpoS^-$ were grown to stationary phase. This experiment did not follow the precise experimental conditions described above. Therefore, the overall range of survival for the WT cells was lower than above results. The cellular density of the cultures was 1.3×10^9 cells/ml instead of the standard 1.8-2.0 $\times 10^9$ cells/ml. This placed the cells in early stationary growth instead of late stationary growth. The cultures were grown in liquid MinA media. 5.0×10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were spread on LB plates and incubated over night. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 120 J/m^2 for the 3 separate cultures.

The wild type strain had 100 fold increase in viability compared to both the PPK^{--} and $RpoS^{--}$ strains, and the difference between PPK^{--} and $RpoS^{--}$ strains was not significant (Figure 22). These results indicate that RpoS does affect the survival after DNA damage, and there still could be a connection between polyphosphates, RpoS, and the reduced viability after DNA damage. However, the reduction in survival in $RpoS^{--}$ cells may be due to the transcription functionality. The SOS genes do not have a known promoter region preferring one transcription factor over another, and, therefore, the reduce viability may be caused by the reduced number of transcription factors for SOS activation.

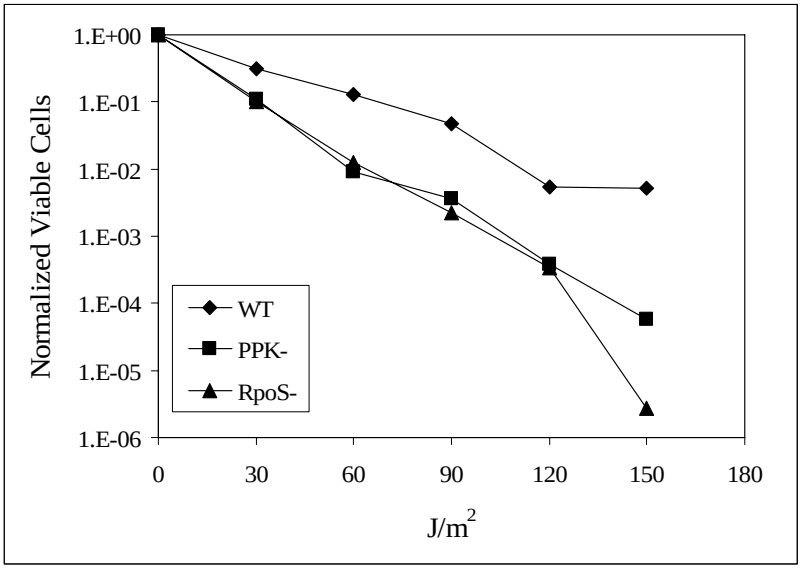


Figure 22 – Are cells without RpoS sensitive to DNA damage?

WT and PPK$^-$ cells are designated with ◆ and ■, respectively. RpoS$^-$ cells are designated by ▲. Cells are grown in MinA minimal media to early stationary phase overnight at 37 °C to a concentration of 1.3 x 10^9 cells/ml and exposed to 254 nm UV light at 90 J/m^{2}min. The viability was determined by plating the radiated samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed in triplicate. The experiment was proved statistically significant using the Student t-test comparing WT and RpoS$^-$, and PPK$^-$ and RpoS$^-$ at 120 J/m^2. $p = < 0.05$, NS, respectively.

Do reduced PPK or polyphosphate quantities cause the decreased viability of RpoS$^-$ cells?

In previous studies, reduced polyphosphate levels did not increase in strains without RpoS (Ault-Riche et al., 1998). If polyphosphate levels were reduced in the RpoS$^-$ cells used above, the reduced viability may be caused by the same reason reduced polyphosphate levels decrease survival. To test this hypothesis, PPK was overproduced in RpoS$^-$ cells to determine if polyphosphates would rescue these cells.

RpoS$^-$ cells were transformed with the pPPK plasmid and the pVector plasmid. The survival phenotype was compared between WT, PPK$^-$, RpoS$^-$, RpoS$^-$ /pPPK, and RpoS$^-$/pBAD cells. This experiment did not follow the precise experimental conditions described above, therefore the overall range of survival for the WT cells was lower than the above results. WT, PPK$^-$, RpoS$^-$, RpoS$^-$/pPPK, and RpoS$^-$/pBAD cultures were grown overnight to a density of 1.3 x 10^9 cells/ml instead of the standard 1.8-2.0 x 10^9 cells/ml. This placed the cells in early stationary growth instead of late stationary growth. The cultures were grown in liquid MinA media containing succinate instead of glucose as the carbon source. One hour prior to UV radiation, the plasmids were induced by introducing 0.04% arabinose to the cultures. After continuing the incubation for one hour, the cultures were diluted to 5.0 x 10^9 cells in 20 ml of 100 mM $MgSO_4$ and were exposed to 254 nm UV light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were spread on LB plates and incubated overnight. CFU

were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 120 J/m^2 for the 3 separate cultures.

The decreased survival phenotype of the RpoS^{--} strain was not able to be rescued with excess polyphosphates generated by transforming a plasmid carrying a copy of the *ppk* gene (pPPK). There was no significant difference between PPK$^-$, RpoS^{--} , RpoS$^-$/pPPK, and RpoS$^-$/pBAD (Figure 23). This concludes that the decrease in viability following DNA damage for the RpoS^{--} strain was not caused by low polyphosphate levels.

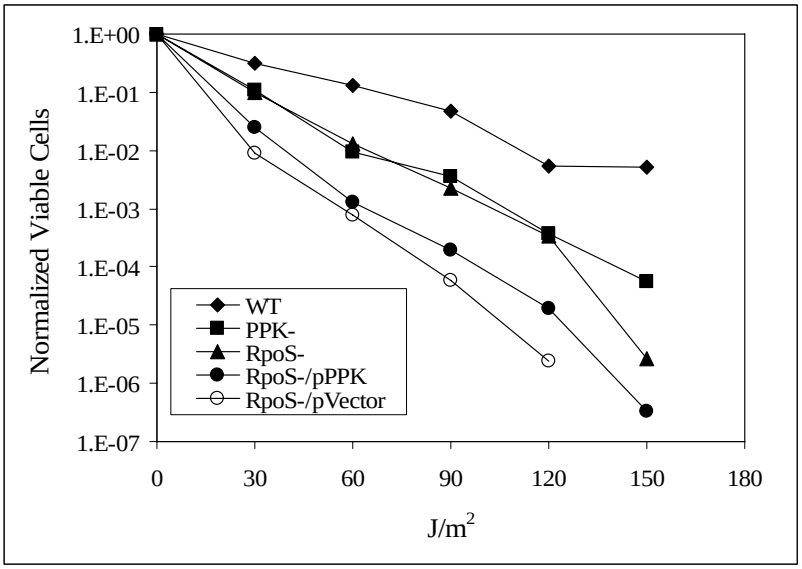


Figure 23 – Do reduced PPK or polyphosphate quantities cause the decreased viability of RpoS^{--} cells?

WT and PPK$^-$ cells are designated with ◆ and ■, respectively. RpoS$^-$ cells are designated by ▲ and RpoS$^-$ cells containing plasmid with and without *ppk* gene are designated by ● and ○, respectively. Cells are grown in MinA minimal media to stationary phase overnight at 37 °C to a concentration of 1.3 x 10^9 cells/ml and exposed to 254 nm UV light at 90 J/m^{2}min. The viability was determined by plating the radiated samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed in triplicate. The experiment was proved statistically significant using the Student t-test comparing WT and RpoS$^-$ /pPPK, WT and RpoS$^-$/pVector, PPK$^-$ and RpoS$^-$/pPPK, PPK$^-$ and RpoS$^-$/pVector, RpoS$^-$ and RpoS$^-$ /pPPK, RpoS$^-$ and RpoS$^-$/pVector, at 120 J/m^2. p values are <0.05, <0.05, NS, NS, NS, and NS, respectively.

Are the levels of polyphosphates different in RpoS^{--} cells?

To confirm the above conclusion that the lack of RpoS in cells does not cause reduced polyphosphates resulting in decreased survival, polyphosphate levels were measured in WT, PPK$^-$, and RpoS$^-$ cells. The cultures were grown in MinA media to stationary phase as

described. The cultures were diluted to 5.0 x 10^9 cells in 20 ml of 100 mM $MgSO_4$ prior to exposure to 150 J/m^2 254 nm UV light in a 100 mm petri dish. The cells were concentrated back to the original optical density and left to incubate at 37 °C for a defined time. The cells were then removed, placed on ice and lysed in 4M GITC. The polyphosphates were extracted and measured for these samples as described (methods). Figure 24 compares the levels of polyphosphates for samples with no UV, one hour after UV exposure, and four hours after exposure. The assay was performed in triplicate and the average value and standard deviations are illustrated.

The RpoS⁻⁻ strain contained polyphosphate levels at similar levels to WT prior to radiation while the PPK⁻ strain contained minimal amounts. PPK⁻ levels remained low after exposure, while WT and RpoS⁻⁻ polyphosphate levels elevated within one hour and reduced within four hours (Figure 24). RpoS⁻⁻ cells showed an increased elevation of polyphosphates over WT, but this increase was not statistically significant at one hour after radiation according to the Student T test, p= 0.08. The increased elevation over WT in the RpoS⁻⁻ cells still appeared at four hours after exposure with a p value of 0.008. There is not enough evidence to determine if RpoS⁻⁻ cells constantly contain higher quantity of polyphosphates or if this elevated amount has any effect on the RpoS⁻⁻ survival phenotype. It has been confirmed through these experiments that the decreased survival of PPK⁻⁻ cells after DNA damage is not dependent of RpoS.

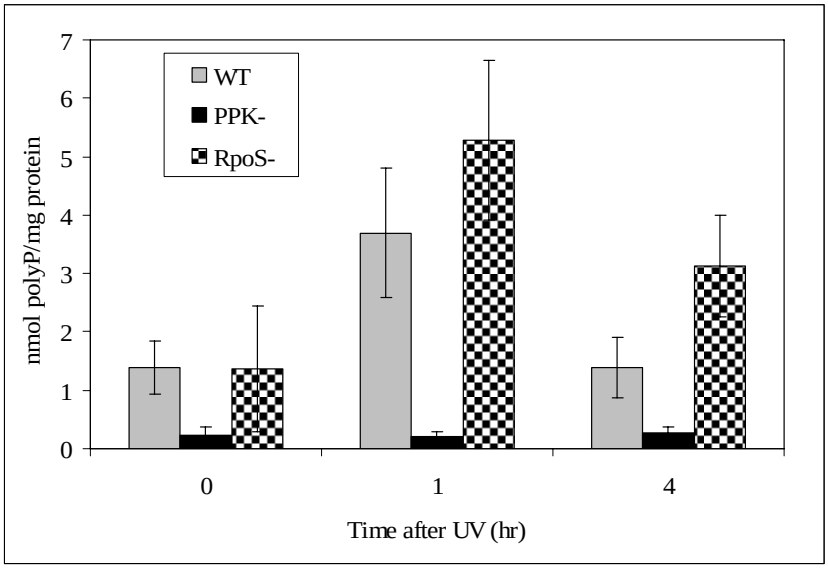


Figure 24 – Are the levels of polyphosphates different in RpoS⁻⁻ cells?

WT, PPK⁻, and RpoS⁻ cells are designated with □ , ■ and ▨ , respectively. Cells are grown in MinA minimal media to stationary phase at 37 °C and exposed to 254 nm UV light for 150 J/m^2. Cells were returned to 37 °C to incubate for 1 and 4 hours. The cells were lysed and the polyphosphates collected and measured. The experiment was performed in triplicate. Values displayed are averages with the standard deviations.

Epistatic Studies Between *ppk* and SOS genes

Removal of two genes from a genome can give varying phenotypic differences compared to single gene deletions. Four qualitatively distinct situations may arise when analyzing the sensitivity of a double mutant strain: 1) The sensitivity may be equal to that of the more deficient single mutant parent. 2) The sensitivity may have an additive effect when it is equal to the sum of each of the single mutant parents. 3) A synergistic effect is the result when the sensitivity is greater than the sum of the single mutant parents. 4) Finally, in rare situations, the sensitivity may be less than one of the single mutant parents, indicating an activation effect.

Such an epistatic study was carried out between the gene encoding PPK and five genes for proteins known to be involved in the SOS response. UvrA participates in the NER mechanism. RecA, a core SOS, recognizes and binds single stranded DNA that serves as a signal of DNA damage, thereby inducing the SOS response. RecA also activates cleavage of LexA and UmuD, participates in DNA recombination repair, and participates in Pol V polymerase activity. DNA polymerases II, IV, and V all are induced by the SOS response. While Pol II restarts replication after error free repair, Pol IV and Pol V are error prone polymerases, which are able to replicate pass damaged DNA.

Is ppk epistatic to uvrA?

DNA repair mechanisms are the key components for cell survival following DNA damage. It was shown that polyphosphates participate in the survival of cells following severe DNA damage but it was not shown if polyphosphates participate in the NER mechanism directly. To test this possible association of polyphosphates and the NER mechanism, an epistatic experiment was performed between *ppk* and *uvrA*.

Cells without *ppk* (PPK$^-$), *uvrA* (UvrA$^-$), or *ppk* and *uvrA* (PPK$^-$/UvrA$^-$) were grown to stationary phase overnight following the standard experimental conditions described (Methods). The cultures were grown in liquid MinA media to stationary phase. 5.0×10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were spread on LB agar and incubated overnight. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 30 J/m^2 for 4 separate cultures resulting with a p value < 0.0005

UvrA epistatic studies show UvrA knockout cells are extremely sensitive to UV radiation and have only 0.01% survival after exposure to 30 J/m^2 (Figure 25). This severe sensitivity to UV prevented detection of a difference between the UvrA$^-$ and the PPK$^-$/UvrA$^-$ cells.

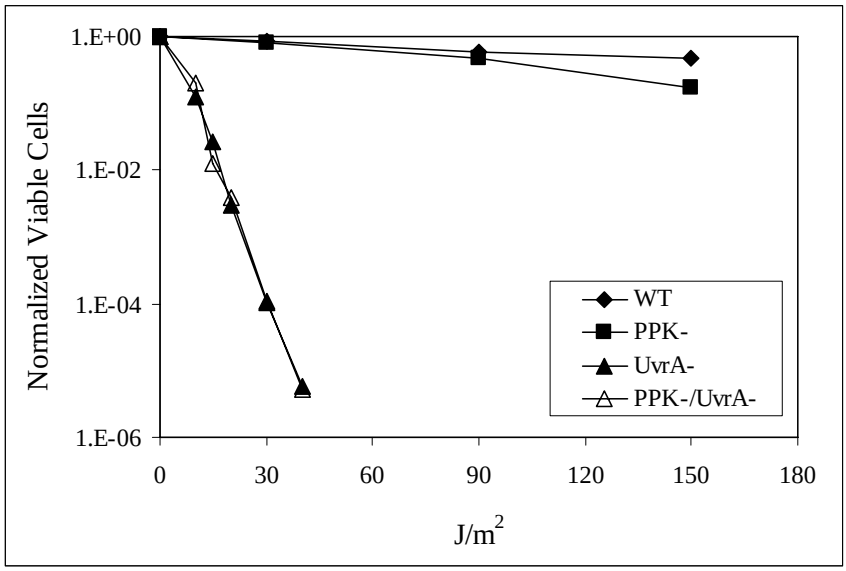


Figure 25 – Is *ppk* epistatic to *uvrA*?

WT and PPK⁻ cells are designated with ◆ and ■, respectively. UvrA⁻ cells are designated by ▲ and cells without UvrA and without PPK are designated by △. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and exposed to 254 nm UV light at 90 J/m^{2}min. The viability was determined by plating the samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed in triplicate.

Is ppk epistatic to recA?

RecA controls the SOS response at many different points in the pathway. RecA signals the need for the SOS response, controls the release of LexA from SOS promoters, controls the activity of Pol V through UmuD cleavage and polymerase activity, and participates in recombination DNA repair. Since it is at the core of the SOS response, an epistatic experiment was performed between *ppk* and *recA.*

Cells without *ppk* (PPK⁻), *recA* (RecA⁻), or *ppk* and *recA* (PPK⁻/RecA⁻) were grown to stationary phase overnight following the standard experimental conditions described (Methods). The cultures were grown in liquid MinA media to stationary phase. 5.0 x 10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were spread on LB agar and incubated overnight. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 120 J/m^2 for 3 separate cultures.

Cells that do not have the RecA protein are substantially more sensitive than wild type or PPK⁻⁻ cells (Figure 26). The double knockout cells appear to be even more sensitive, yet statistically this difference was not significant. Due to the extreme difference in sensitivity between RecA⁻ cells and PPK⁻ cells, it was difficult to accurately define their epistatic relationship. Polyphosphates and RecA may participate in different pathways, with both

affecting the cells ability to survive. However, it is more likely that polyphosphates and RecA participate in some common pathways, since RecA is involved in so many of the SOS mechanisms.

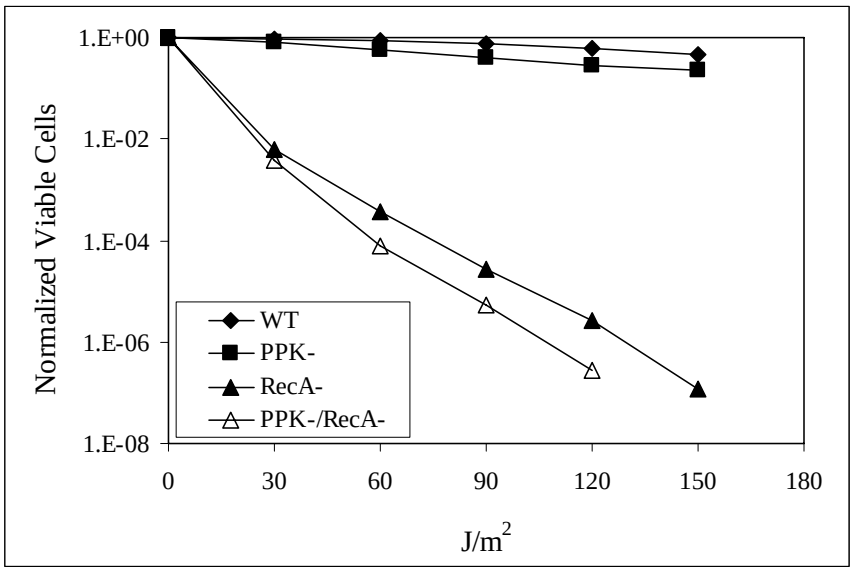


Figure 26 – Is *ppk* epistatic to *recA*?

WT and PPK⁻ cells are designated with ◆ and ■, respectively. RecA⁻ cells are designated by ▲ and cells without RecA and without PPK are designated by △. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and exposed to 254 nm UV light at 90 J/m^{2}min. The viability was determined by plating the samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed in triplicate. The experiment was proved statistically significant using the Student t-test comparing WT and RecA⁻, PPK⁻ and RecA⁻, PPK⁻ and RecA⁻/ PPK⁻, RecA⁻ and RecA⁻/ PPK⁻ cells at 120 J/m^2. p = < 0.005, < 0.005, < 0.05, NS, respectively.

Is ppk epistatic to polB?

The SOS response triggers the transcription of Pol II immediately after activation. Pol II participates in error-free TLS by helping Pol III to restart and bypass the DNA lesion. Pol II was identified to represent an early onset SOS gene to determine an epistatic relationship with *ppk*.

Cells without *ppk* (PPK⁻), *polB* (Pol II⁻), or *ppk* and *polB* (PPK⁻/Pol II⁻) were grown to stationary phase overnight following the standard experimental conditions described (Methods). The cultures were grown in liquid MinA media to stationary phase. 5.0 x 10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were spread on LB agar and incubated overnight. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 150 J/m^2.

Knockouts of the *polB* gene were previously published to have minimal increased susceptibility to UV when compared to wild type cells (Bhattacharya and Beck, 2002). Therefore, it was not surprising to find that the Pol II$^-$ cells have the same sensitivity to UV as wild type cells, and the double knockout cells show the same survival phenotype as PPK^{--} cells (Figure 27).

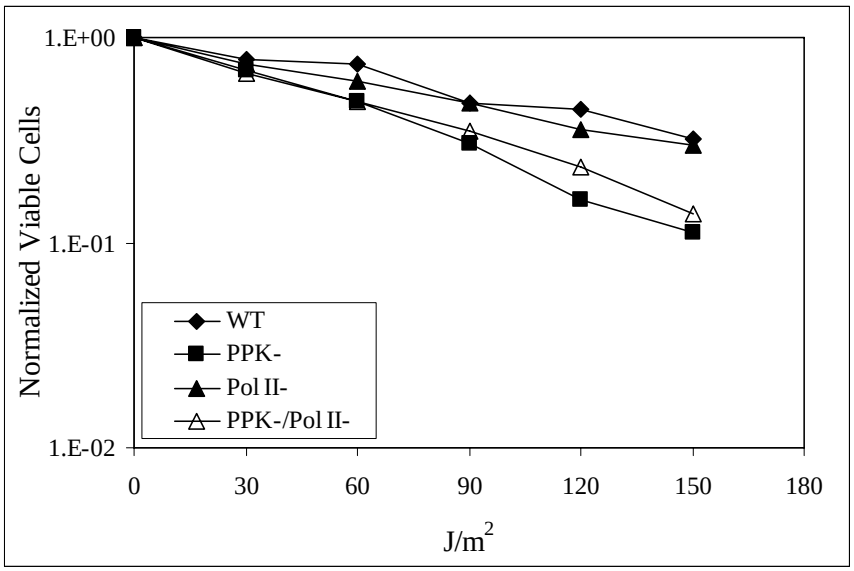


Figure 27 – Is *ppk* epistatic to *polB*?

WT and PPK$^-$ cells are designated with ◆ and ■, respectively. Pol II$^-$ cells are designated by ▲ and cells without Pol II and without PPK are designated by △. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and exposed to 254 nm UV light at 90 J/m^{2}min. The viability was determined by plating the samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed in triplicate. The experiment was proved statistically significant using the Student t-test comparing WT and Pol II$^-$, PPK$^-$ and Pol II$^-$, PPK$^-$ and Pol II$^-$/ PPK$^-$, Pol II and Pol II$^-$/ PPK$^-$ cells at 120 J/m^2. p = NS, < 0.005, NS, < 0.005, respectively.

Is ppk epistatic to umuDC?

Pol V is a key error-prone polymerase that is activated in phase two of the SOS activation. If the cell is unable to repair itself with error-free methods, it then activates the error-prone TLS as a last chance to stay alive. It was reported that Pol V is the main error-prone polymerase to tackle UV DNA damage. To test if Pol V and polyphosphates interrelate in the SOS response, an epistatic study was performed between *ppk* and *umuDC*.

Cells without *ppk* (PPK$^-$), *umuDC* (Pol V$^-$), or *ppk* and *umuDC* (PPK$^-$/Pol V$^-$) were grown to stationary phase overnight following the standard experimental conditions described (Methods). The cultures were grown in liquid MinA media to stationary phase. 5.0 x 10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were spread on LB agar and incubated

overnight. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 180 J/m^2 for 3 separate cultures in each experiment and over 3 separate days of experiments.

The epistatic analysis of PPK and Pol V detected potential participation in different pathways for polyphosphates and Pol V (Figure 28). Cells without Pol V were more sensitive than wild type or PPK$^-$ cells (Figure 28). The double knockout cells appeared to be even more sensitive, with an additive increase in sensitivity. This indicates that polyphosphates and Pol V may participate in different pathways, with both affecting the cells ability to survive.

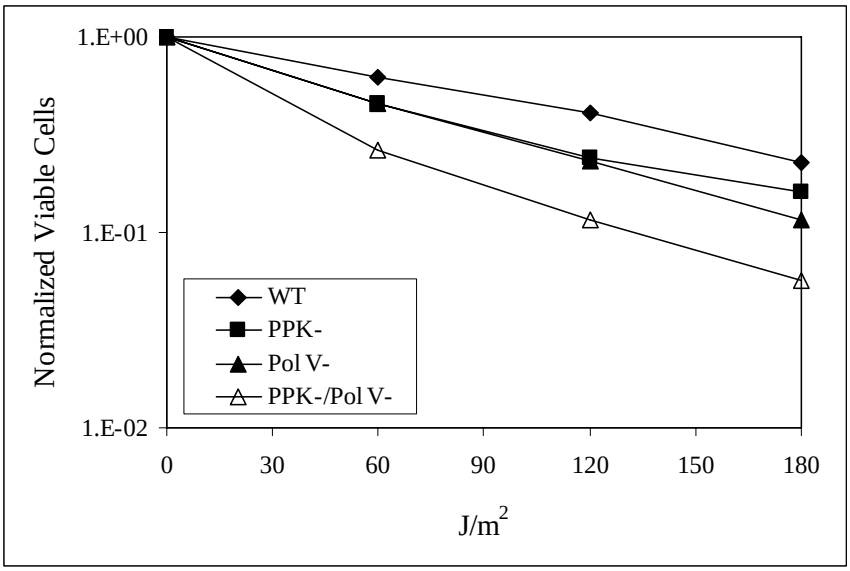


Figure 28 – Is *ppk* epistatic to *umuDC*?

WT and PPK$^-$ cells are designated with ◆ and ■, respectively. Pol V$^-$ cells are designated by ▲ and cells without Pol V and without PPK are designated by △. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and exposed to 254 nm UV light at 90 J/m^{2}min. The viability was determined by plating the samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed 3 times in triplicate, n = 9. The experiment was proved statistically significant using the Student t-test comparing WT and Pol V$^-$, PPK$^-$ and Pol V$^-$, PPK$^-$ and Pol V$^-$/ PPK$^-$, Pol V and Pol V$^-$/ PPK$^-$ cells at 120 J/m^2. p = <0.005, NS, <0.005, <0.005, respectively.

Is ppk epistatic to dinB?

Pol IV is the second error-prone polymerase that is activated in phase two of the SOS activation. Pol IV's activation is less complicated than Pol V. Pol V requires RecA to activate autocleavage of UmuD, Pol V is a hetero-trimer of 2 UmuD' and 1 UmuC, and it requires RecA for activity at the replication fork. The only known substrate needed for Pol IV activity is the β-clamp which is required by all DNA polymerases. To test if Pol IV and polyphosphates interrelate in the SOS response, an epistatic study was performed between *ppk* and *dinB*.

Cells without *ppk* (PPK⁻), *dinB* (Pol IV⁻), or *ppk* and *dinB* (PPK⁻/Pol IV⁻) were grown to stationary phase overnight following the standard experimental conditions described (Methods). The cultures were grown in liquid MinA media to stationary phase. 5.0×10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were spread on LB agar and incubated overnight. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 150 J/m^2 for 3 separate cultures in each experiment and over 3 separate days of experiments.

Comparison of survival curves of cells with and without Pol IV and PPK indicated that the double knockout cells have the same survival phenotype as the single PPK knockout cells (Figure 29). This was not expected since Pol IV is another error prone polymerase like Pol V. They both belong to the same protein family and perform similar functions in the SOS response. Interestingly, the Pol IV single knockout cells have even greater sensitivity than the Pol IV and PPK double knockout cells. This suggests polyphosphates play a role, directly or indirectly, in Pol IV activity. Without PPK, the effect on survival caused by the loss of Pol IV is not realized by the cell. The conclusion will analyze this unique result to the epistatic assay.

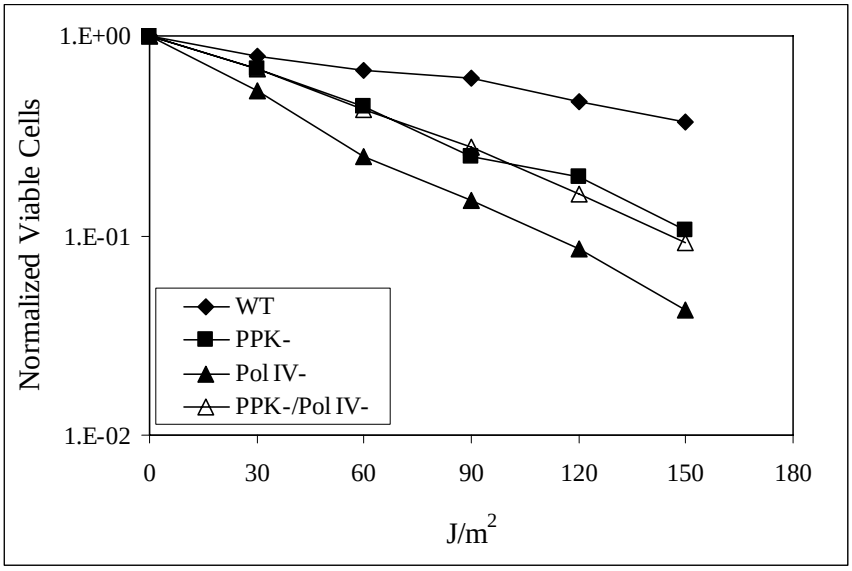


Figure 29 – Is *ppk* epistatic to *dinB*?

WT and PPK⁻ cells are designated with ◆ and ■, respectively. Pol IV⁻ cells are designated by ▲ and cells without Pol IV and without PPK are designated by △. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and exposed to 254 nm UV light at 90 J/m^{2}min. The viability was determined by plating the samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed in triplicate 3 times, n=9. The experiment was proved statistically significant using the Student t-test comparing WT and Pol IV⁻, PPK⁻ and Pol IV⁻, PPK⁻ and Pol IV⁻/ PPK⁻, Pol IV and Pol IV⁻/ PPK⁻ cells at 120 J/m^2. p = <0.0005, <0.005, NS, <0.005, respectively.

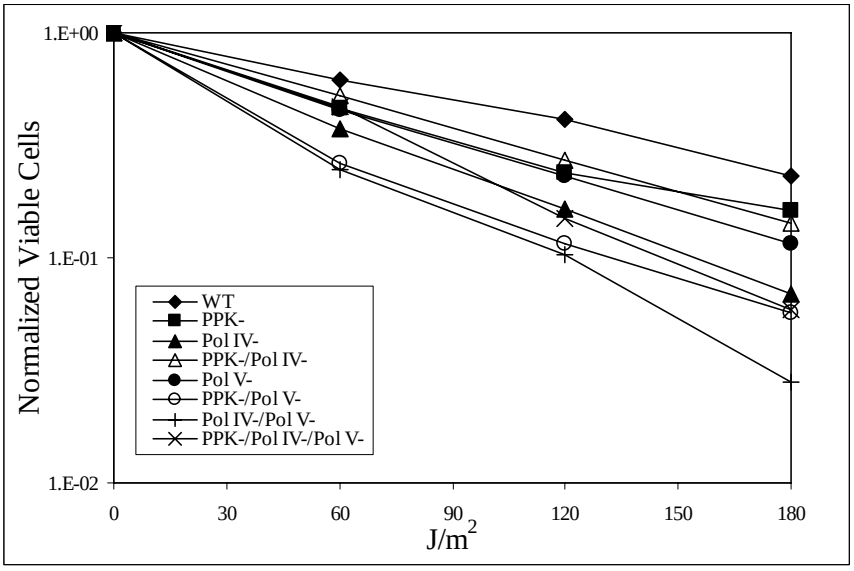
Is ppk epistatic to umuDC and dinB?

In the results described above, it was shown that the two error-prone polymerases had different *ppk* epistatic results. This suggests that polyphosphates play a particular role with one of these polymerases that differs from the other. Analyzing the data led to the question how would the survival curves appear with a triple knockout of PPK⁻/Pol V⁻/Pol IV⁻? Would the removal of polyphosphates from the double error-prone polymerase knockout cause a result like PPK⁻/Pol V⁻, which showed an additive sensitivity, or a result like PPK⁻/Pol IV⁻, which showed an increase in viability without polyphosphates? To test this, an epistatic study was performed between *ppk, dinB,* and *umuDC*.

Cells without *ppk* (PPK⁻), *dinB* (Pol IV⁻), *ppk* and *dinB* (PPK⁻/Pol IV⁻), *umuDC* (Pol V⁻),*ppk* and *umuDC* (PPK⁻/Pol V⁻), and *ppk, dinB* and *umuDC* (PPK⁻/Pol IV⁻/Pol V⁻) were grown to stationary phase overnight following the standard experimental conditions described (Methods). The cultures were grown in liquid MinA media to stationary phase. 5.0×10^9 cells in 20 ml of 100 mM $MgSO_4$ were exposed to 254 nm UV light in a 100 mm petri dish. Samples were gathered at designated UV quantities. The samples were spread on LB agar and incubated overnight. CFU were counted. The data were normalized to 0 J/m^2 UV light, and the average was calculated for the three separate cultures of the strain. To determine the statistical significance, the Student T test was performed comparing the normalized survival value of the strains at 180 J/m^2 for 3 separate cultures in each experiment and over 4 separate days of experiments.

The knockout of the two error-prone polymerases had the greatest sensitivity out of all eight strains in figure 30. Removing PPK caused the viability to increase as it did with the PPK⁻/Pol IV⁻ strain. This suggests that polyphosphates, or PPK, may: 1) Partially inhibit some forms of repair other that Pol IV TLS, 2) Assist in Pol IV initiation and blocks DNA repair without the presence of Pol IV.

A.



B

180 J/m2 p value, n=12

	WT	PPK-	Pol IV-	PPK- / Pol IV-	Pol V-	PPK- / Pol V-	Pol IV- / Pol V	PPK- / Pol IV- / Pol V-
WT								
PPK-	< 0.005							
Pol IV-	< 0.0005	< 0.05						
PPK- / Pol IV-	< 0.0005	NS	< 0.0005					
Pol V-	< 0.0005	NS	< 0.05	NS				
PPK- / Pol V-	< 0.0005	< 0.005	< 0.05	< 0.005	< 0.005			
Pol IV- / Pol V	< 0.0005	< 0.005	< 0.0005	< 0.0005	< 0.0005	< 0.05		
PPK- / Pol IV- / Pol V-	< 0.0005	< 0.05	NS	< 0.05	< 0.05	NS	< 0.005	

Figure 30 – Is *ppk* epistatic to *umuDC* and *dinB*?

WT and PPK$^-$ cells are designated with ◆ and ■, respectively. Pol IV$^-$ cells are designated by ▲ and PPK$^-$/Pol IV$^-$ cells are designated by △. Pol V$^-$ cells are designated by ● and PPK$^-$/Pol V$^-$ cells are designated by ○. Pol IV$^-$/Pol V$^-$ cells are designated by ＋ and PPK$^-$/Pol IV$^-$/Pol V$^-$ cells are designated by ×. Cells are grown in MinA minimal media to stationary phase overnight (~20 hrs) at 37 °C and exposed to 254 nm UV light at 90 J/m^{2}min. The viability was determined by plating the radiated samples on LB agar. Percent survival is determined as the viable cell number at each radiation quantity divided by the viable cell number without radiation. The experiment was performed in triplicate 4 times, n=12. The experiment was proved statistically significant using the Student t-test. The p values are listed in B.

Polyphosphate Levels of SOS Knockouts

Are the polyphosphate levels affected by SOS proteins?

Studies that analyzed polyphosphates and the general stress response reported mutations of stress proteins, RpoS, RpoN, RelA and PhoB, caused a decrease in the polyphosphate accumulation after an applied stress (Ault-Riche *et al.*, 1998; Rao and Kornberg, 1996). It was not known if any of the SOS proteins analyzed in this study caused the same effect on the polyphosphate levels. To examine this, polyphosphate levels were measured on strains mutated of a SOS protein after the cells were exposed to 150 J/m^2 254 nm UV light.

The cultures of the strains were grown and radiated in the same fashion described. The cultures were grown in MinA media to stationary phase. After exposure, the cells were concentrated back to the original optical density and left to incubate at 37°C. Samples were collected at time points ranging from 30 minutes to 5 hours. The polyphosphates were extracted and measured for these samples as described (methods). Figure 31 compares the levels of polyphosphates for samples one hour after UV exposure. One hour after exposure is the approximate peak of polyphosphates after UV radiation (figure 21). The assay was performed in triplicate and the average value and standard deviations are illustrated.

The cellular polyphosphate levels following UV radiation equaled wild type levels for cells without RecA, Pol IV, or Pol V. This was over 10 fold increase compared to PPK$^-$ cells. The PPK$^-$ cells contained minimal amounts of polyphosphates as seen in figure 21. The data support what is known about the SOS response. The error prone polymerases are at the end of the DNA repair pathway. They are transcribed during phase two of the SOS response and are the last opportunity for DNA repair and cell survival. RecA is a more complex protein in the SOS response. It is involved throughout the entire process; initiating the response, activating

downstream proteins and activating the repair mechanisms. Based upon the polyphosphate levels, RecA also does not cause a change in polyphosphate concentration.

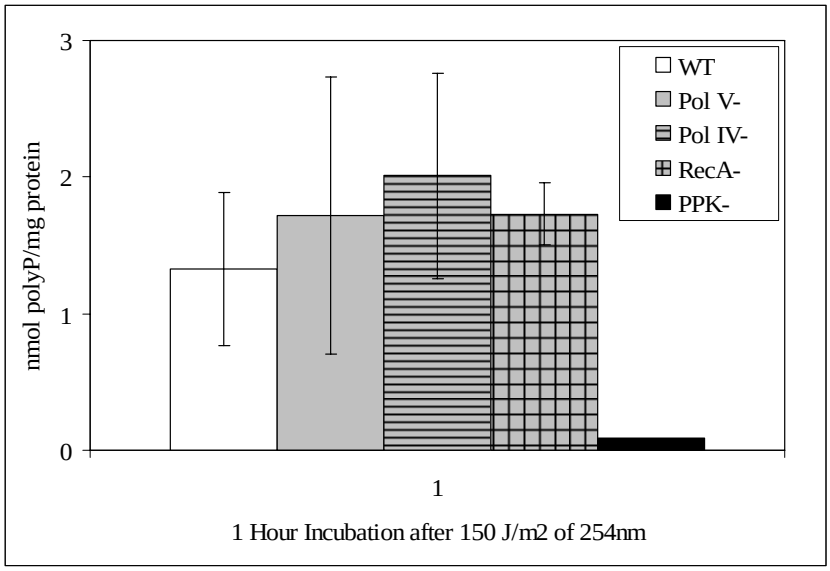


Figure 31 – Are the polyphosphate levels affected by SOS proteins?

WT, PPK$^-$, Pol V$^-$, Pol IV$^-$, and RecA$^-$ cells are designated with □, ■, ▒, ▤, and ▦, respectively. Cells are grown in MinA minimal media to stationary phase at 37 °C and exposed to 254 nm UV light for 150 J/m^2. Cells were returned to 37 °C to incubate after exposure for 1 hour. The cells were lysed and the polyphosphates collected and measured. The experiment was performed in triplicate. Values displayed are averages with the standard deviations.

Methods

Bacterial Strains and Plasmids

The strains and plasmids used in these experiments are listed in table 1.

Red Cloning

To insert foreign DNA into the genome of the *E. coli,* the RED cloning technique was utilized (Yu *et al.*, 2000). The procedure included three steps, preparing insert, preparing electrocompetent cells, and transformation.

First, PCR primers were designed to flank the cassette that would be entered into the desirable target gene. The primers were designed to have overhangs of 35 to 50 bp which were homologous to a portion of the target DNA along with 20 bp which primed the cassette DNA for amplification (see figure 4). The PCR reaction was performed and the amplified product was purified from a 0.8% agarose gel in TAE using Qiagen's gel purification kit (Sambrook *et al.*, 1989). The amplified cassette with homologous flanks was prepared for insertion into a RED expression strain, DY330.

Second, DY330 cells were made competent for electroporation by growing the cells overnight in LB media at 32° C, DY330 is temperature sensitive. Fresh LB of 50 ml was inoculated by the overnight culture and grown at 32° C to an OD_{600} = 0.5 to 0.8. 10 ml of the culture was induced in a fresh flask by placing the flask in a 42° C shaking water bath for 15 minutes. The culture was immediately cooled in an ice bath for 30 minutes. The cells were centrifuged for 8 minutes at 5500g into a pellet at 4° C and washed with 1 ml ice cold water three times. The cells were finally resuspended in 100 µl ice cold water and were ready for electroporation.

Third, 100 ng of PCR product was mixed with 50 µl of DY330 competent cells. The solution was transferred to a 0.1 cm electroporation cuvette. The cells were electroporated with the DNA at 1.8 kV. 1 ml of LB media was added to the cuvette and transferred to a 1.5 ml tube for incubation at 32° C for 1.5 hours. This allows time for the selectable marker to be expressed prior to spreading the cells on selective plates. The plates were incubated overnight at 32° C for colonies to grow. Colonies were selected and screened for the appropriate insert. P1 transduction was used to transfer the gene from the DY330 background to the preferred strain.

P1 Transduction

P1 bacteriophage was utilized to transfer portions of genomic DNA from one *E. coli* strain, donor, into another, host, according to Miller's procedure with minor modifications (Miller, 1972). P1 lysate was grown on the donor strain. The donor strain was grown to exponential phase in LB media with 100 mM $MgCl_2$ and 5 mM $CaCl_2$. At this time 50 µL of phage with a multiplicity of infection (moi) of around 0.1 was added to 100 µL of donor culture. 3 ml of top-agar containing 5 mM $CaCl_2$ was heated to 40 °C and added to the culture/phage prior to pouring onto LB plates containing 5 mM $CaCl_2$. The plates were incubated overnight at 30 °C to allow the phage to infect and lyse the cells. During this process portions of the host strain genome was replicated and incorporated into the phage genome prior to cell lysis. To harvest the phage, the

top agar was scrapped off the plate. The plate was washed with 1 mL SM buffer and the wash was added to the top agar. 1 mL of chloroform was added to the mix, vortexed vigorously and centrifuged for 15 min at 3500g. The supernatant, P1 phage lysate, was transferred to a fresh tube with 1 mL chloroform to be stored at 4 °C.

To transduce the donor DNA to the host strain using the fresh phage, the host strain was grown overnight in 5 mL of LB. The culture was centrifuged to pellet the cells and the cell pellet was resuspended in 1 mL LB media with 100 mM $MgCl_2$ and 5 mM $CaCl_2$. 50 μL of P1 lysate diluted to a titer of $10^{-9} – 10^{-10}$ PFU/μL was added to the cells before incubating at 37 °C for 20 minutes. 1 mL of 1 M sodium citrate and 2 mL of SOC media were added to the solution, mixing after each reagent. The cells are incubated in the liquid media for 1 hour at 37 °C. The cells were pelleted and resuspended in 1 mL of LB before plating 100 μL aliquots on selectable plates. Only cells with the selectable marker transduced from the donor strain grew into colonies on the plates. The plates were incubated overnight at 37 °C. Colonies were selected, restreaked on selectable plates, and grown in LB liquid media containing appropriate antibiotics, see table 1. The new strains were confirmed using PCR screening.

Polymer Chain Reaction - PCR Screening

The new strains were confirmed using PCR screening. PCR was performed according to (Sambrook *et al.*, 1989). PCR primers were designed to span the specified gene. The primers generated PCR products of different sizes for wild type versus interrupted genes. The PCR products were combined with DNA buffer and analyzed on a 1% agarose electrophoresis gel in TAE. The Results describe the genes and PCR primers.

Plasmid Transformation

To transform plasmids into strains, calcium chloride competent cells were used. The procedure was according to (Sambrook *et al.*, 1989). The cells to obtain the plasmid were grown in LB to exponential growth. 25 mL of culture was placed in cold tubes, chilled on ice for 10 minutes and centrifuged to form a cell pellet. The cell pellet was resuspended in 5 mL of ice-cold 0.1M $CaCl_2$ and left on ice for ten minutes before centrifuged to form another cell pellet. After resuspending the second cell pellet in 1 mL of ice-cold 0.1M $CaCl_2$, 200 μL aliquots of these competent cells were used for the transformation of the plasmid. 50 ng of plasmid DNA was added to the competent cells and gently mixed. After sitting on ice for 30 minutes, the cells were warmed to 42 °C for 90 seconds and returned to ice for 2 minutes. 800 μL of LB was added to the cells for a total of 1 mL. The suspension was incubated at 37 °C for 1 hour. 200 μL of transformed cells were spread on selectable plates and incubated overnight at 37 °C. Colonies were selected, grown in liquid media with appropriate antibiotic, and screened for the plasmid.

Media and growth conditions

Cultures were either grown in MinA minimal media (Miller, 1972) supplemented with 1 mM $MgS0_4$, 0.2% carbon, 1μg/mL thiamine, and 50 μg/mL thymine, or in a rich medium, Luria-Bertani (LB) liquid medium (Miller, 1972). The carbon source for all cultures was glucose except for the strains with the inducible plasmids pBAD or pPPK. These cultures were grown with 0.2% succinate until induced with 0.4% arabinose. All cultures were grown at 37 °C unless

otherwise indicated. Appropriate antibiotics were placed into the growing cultures as required by the antibiotic resistance of the strain or plasmid.

Protein Concentration Determination

Protein concentrations were measured using the Bradford method as described in (Sambrook *et al.*, 1989). 1 mg/ml BSA solution was used as a standard solution. A linear standard curved was formed using 0, 2, 4, 6, 8, and 10 μL of the 1 mg/mL BSA solution with H_2O to form a total of 100 μL of standard sample. Different concentrations of protein samples in a total of 100 μL of a defined buffer were also prepared. 1 mL of Bradford solution was added to each standard and protein samples, the samples were mixed and the absorbance at 595nm wavelength is measured. Protein concentrations were determined based on the standard curve.

PPK Purification

PPK was purified following the protocol described in (Akiyama *et al.*, 1992). Unless otherwise noted, this protocol was performed at 4°C.

W3110/pBC10 cells were grown in ten liters of LB media with 50μg/ml ampicillin at 30°C until the optical density at 600 nm wavelength (OD_{600}) reached 0.05. The culture continued to grow at 37°C for four hours in a Microferm Fermenter by New Brunswick Scientific Co, Inc. When the OD_{600} reached 1.4, the cells were harvested and centrifuged into cell pellets. The pellets were resuspended in equal weight of cold 50 mM Tris-HCl pH 7.5 and 10% sucrose before they were frozen in liquid nitrogen and stored at -80°C. Four cultures were grown to obtain a total of 300 g of cell suspension.

Once cells were frozen and stored, 300 g of cell suspension were thawed on ice. Once completely thawed, 2mM DTT and 250 μg/ml lysozyme were added to the suspension, mixed, and transferred to cold Beckman Ti45 centrifuge tubes. The cells were incubated in the tubes at 0°C for 30 min before they were exposed to 37°C in a circulating water bath for 4 minutes to lyse the cells without shearing. They were immediately chilled in ice water. The suspension was then centrifuged in a Ti45 rotor at 20,000 rpm for 1 hour to collect the particulate fraction. This was defined as fraction I supernatant.

To further purify PPK, particulate fraction was sonicated in the presence of 50 mM Tris-HCl, 10% sucrose, 5 mM $MgCl_2$, 10 μg/ml DNase I and 10 μg/ml RNase A. The temperature during sonication remained at or below 10°C. The sample was divided into smaller fractions for best sonication results and was sonicated until the aggregate was fluid.

After sonication solid KCl was added to the final concentration of 1.0 M and a tenth of total the volume of the sample of 1M Na_2CO_3 was added. This mixture was stirred for 30 minutes at 0°C. To remove the debris, it was then centrifuged in a Ti45 rotor at 44,000 rpm for 1 hour. The supernatant was immediately dialyzed four times against 3 liters of Buffer B (0.2 M potassium phosphate (pH 7.0), 10 % glycerol, 1 mM DTT, 1 mM EDTA). This was defined as dialyzed fraction I.

0.2 g/ml solid, finely ground $(NH_4)_2SO_4$ was added to fraction I and stirred for 30 minutes before the supernatant was collected by centrifuging at 23,500 x g for 1 hour. An additional 0.103g/ml $(NH_4)_2SO_4$ was added to the supernatant and stirred on ice for another 30 minutes.

The centrifuge was repeated but this time the pellet was collected. The pellet was dissolved in 110 ml of Buffer B. This was defined as fraction II.

Fraction II was dialyzed four times against 2 liters of Buffer B. This product was defined as dialyzed fraction II. It was applied to an S-Sepharose Fast Flow column (460 ml, 5.7 X 18 cm) equilibrated in Buffer B and equilibrated until the pH and conductivity of the buffer and resin wash were the same. While the dialyzed fraction II is applied to the column, the 200 ml load was collected and saved as well as the 125 ml flow through. The column was washed with 5 column volumes of buffer B before the protein was eluted with Buffer B containing 1M KCl. One hundred 10 ml fractions were collected as the protein was eluted. A PPK activity assay and protein concentration determination was performed on the fractions. The active fractions were pooled and this was defined as fraction III.

Fraction III was further purified by adding 0.35 g/ml solid finely ground $(NH_4)_2SO_4$ and stirring it for 30 minutes on ice. The suspension was centrifuged at 23,500 x g for 1 hour in a SS-34 rotor at 14K rpm. The pellet was collected and dissolved in 50 ml of Buffer B with 1 M KCl. The suspension was then dialyzed three times against 2 liters of Buffer D containing 150mM KCl. The dialyzed solution was centrifuged in a Beckman Ti60 rotor at 40,000 rpm for 30 minutes and the supernatant was applied to a Mono S HR 10/10 column equilibrated with Buffer D containing 150 mM KCl. After the protein was added to the column, the column was washed with 5 column volumes of Buffer D with 150 mM KCl. The protein was then eluted with 15-column volume linear gradient (0.15-0.7 M KCl) in Buffer D. Fractions were collected in 1.9 ml fractions and assayed for PPK activity and protein concentration. The active fractions were pooled and this was defined as fraction IV, the final purified product.

PPX Purification

scPPX was purified following the protocol described in (Akiyama *et al.*, 1992). Unless otherwise noted, this protocol was performed at 4°C.

CA10/pTrcPPX1 cells were grown in a Microferm Fermenter by New Brunswick Scientific Co, Inc in ten liters of LB media with 100μg/ml ampicillin at 37°C until the optical density at 600 nm wavelength (OD_{600}) reached 0.64. The culture was induced with IPTG for a final concentration of 0.5 mM. After growing at 37°C for 6 hours, the cells were harvested and centrifuged into cell pellets. The pellets were resuspended in four volumes of lysis buffer (20 mM Tris HCl pH=7.5, 2 mM EDTA) before frozen in liquid nitrogen and stored at -80°C.

The frozen cells were completely thawed on ice before lysed by sonication. The temperature during sonication remained at or below 10°C. The sample was divided into smaller fractions for best sonication results and sonicated until the aggregate was fluid. To remove the debris, it was then centrifuged in a Ti45 rotor at 44,000 rpm for 1 hour. The supernatant was immediately dialyzed four times against 2 liters of Buffer A (10 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 0.05 mM EDTA, 2 mM Beta-Mercaptoethanol, 10% Glycerol) and 100 mM NaCl. This was defined as dialyzed fraction I.

Fraction I was applied to a Ni-nitrilotriacetic acid agarose column (30 ml, 2.5 X 6 cm) (ProBond™ Nickel-Chelating Resin from Invitrogen) equilibrated in Buffer A with 100 mM NaCl. The column was washed with 225 mL Buffer A with 500mM NaCl and then re-equilibrate with Buffer A with 100 mM NaCl. 100 ml samples of wash buffer were collected.

The protein was eluted with a linear imidazole gradient (0 to 90 mM in 300 mL) in Buffer A containing 100 mM NaCl. Fractions of 10 ml were collected during protein elution. PPX activity assay and protein concentration determinations were performed on the fractions. The active fractions were pooled and this was defined as fraction II.

Fraction II was dialyzed four times against 2 liters of Buffer A containing 10 mM KCl. A 15% SDS PAGE Gel was run and the gel was stained with Coomassie stain to determine protein purity after the first column. Dialyzed fraction II was applied to a Mono Q column (5mg of protein/ml of Mono Q) equilibrated with Buffer A containing 10 mM KCl. The column was washed with Buffer A containing 10 mM KCl and the protein was eluted with 15-column volume linear gradient (10 to 500 mM KCl) in Buffer A. Eluted protein was collected in 1 ml fractions. PPX activity assay and protein concentration determinations were performed on the fractions. The active fractions were pooled and this was fraction III.

Fraction III was applied to a hydroxyapatite column (1.5 by 9 cm) equilibrated with Buffer B. The column was washed with 70 ml Buffer B and the protein was eluted with linear potassium phosphate gradient (pH = 7.4, 120 ml, 0 to 200 mM) in Buffer B. Eluted protein was collected in 1 ml fractions. PPX activity assay and protein concentration determinations were performed on the fractions. The active fractions were pooled and are stored at –80 °C.

In Vitro PolyP Synthesis

γ-^{32}P PolyP were synthesized using purified PPK as described in (Ault-Riche *et al.*, 1998). γ^{32}P-ATP was purchased from GE Healthcare. Each reaction produced approximately 35 mM polyP at the length of 750 phosphate units per chain. 1 mM ATP, 50 mM HEPES-KOH pH=7.2, 4 mM $MgCl_2$, 40 mM $(NH_4)_2SO_4$, 2 mM Creatine Phosphate, 4 μg/ml Creatine Kinase, and γ^{32}P-ATP to equal 2,000 – 10,000 CPM/nmol polyP were combined with PPK for a total of 750 μL. It was incubated for 1 hour at 37°C to synthesize the polyP. The reaction was stopped with 70 μL of 0.5 M EDTA pH=8.0. To purify the long chain polyP, 410 μL of sec-butanol was added to each sample to precipitate the polyP while the incubating at -20°C for 1 hour. The samples were micro-centrifuged at high speed at 4°C to gather the precipitated polyP into a pellet. The pellet was dried before being resuspended in 20 μL of 50 mM Tris-HCl pH = 7.4. TLC was performed during and after the reaction to measure the rate of reaction, at the completion of the reaction and at the completion of purification of the product.

Thin Layer Chromatography (TLC)

Polyphosphate size and purity were determined by TLC. When synthesizing polyphosphate with γ-P^{32} ATP, reaction rate, reaction completion, and full length product purity was determined using TLC. PPX activity was also measured by detecting P^{32} polyphosphate degradation.

PEI Cellulose F TLC plates from EMD Chemicals were divided into lanes 1 cm wide and 10 cm tall. The TLC chamber was thoroughly rinsed with dH_2O and a strip of Whatman chromatography paper was placed on the bottom of the tank. The tank was filled with TLC solvent (1M KH_2PO_4 for long chain polyphosphate or 1M LiCl – 0.4M formic acid for PPX reactions) to completely wet and submerse the Whatman paper which laid flat on the bottom of the tank.. The top of the tank was covered with enough grease to firmly seal the lid.

To run the sample, 1-2 μL was placed 1 cm above the bottom of the TLC strip. For a control, 2 μL of 100 mM ATP was placed in a lane beside the sample(s). The TLC plate was left at room temperature for 5 minutes before drying the plate briefly under a heat lamp. Prior to placing the TLC plate into the TLC chamber, the chamber was brought to gas-liquid equilibrium by sealing the lid and leaving the tank at room temperature for 1 hour. When the tank was ready, the bottom edge of the plate was placed on the Whatman paper that was absorbed in the solvent in the tank. The top edge of the plate was leaned against the tank wall. The lid was sealed and the solvent was allowed to migrate to the top of the TLC strip.

The TLC plate was analyzed with a long wavelength UV source to mark the ATP migration point which was the only visible band and located near the top of the strip. After the plate was dried under a heat lamp, the samples were analyzed with either a scintillation counter or with a phosphoimager (Molecular Dynamics STORM 840).

UV Survival Assay

Viability was determined as the percent of colony forming units (cfu) of the treated cultures compared to that of untreated at time 0. Survival of *E. coli* was assayed by growing cells either in MinA minimal media or in LB media to specified growth phase. For experiments with inducible plasmids, one hour before UV exposure the cultures were induced with 0.4% arabinose and left to continue growing at 37°C. The concentration of the cultures was determined using the optical density at 600nm wavelength and normalized to 0.25×10^9 cells/mL in ice cold 1 mM $MgSO_4$ buffer. The cultures remained on ice and without direct visible light throughout the UV exposure. 20 mL of normalized culture was placed in a 100 mm petri dish giving a 2.5 mm depth of culture. The dish sat on packed ice while being exposed to 254 nm UV light by a model UVGL-25 Minerallight® lamp by UVP, Inc. To ensure consistent exposure, the UV was measured with a Spectroline® Digital Radiometer model DRC-100X. At the specified times, 1.0 mL samples were withdrawn from the exposed sample and diluted in LB media. 0.1 mL aliquots were spread on LB plates. The plates were wrapped in foil to prevent visible light exposure and incubated overnight at 37°C. The colonies on the plates were counted and the viable cell counts were calculated.

PolyP Levels after UV Exposure

PolyP levels were determined by following a similar UV exposure procedure as described for the UV Survival Assay. The cultures were grown in MinA minimal media at 37°C to stationary phase. The concentration of the cultures was determined using the optical density at 600nm wavelength and normalized to 0.25×10^9 cells/mL in ice cold 1 mM $MgSO_4$ buffer. The cultures remained on ice and without direct visible light throughout the UV exposure. 20 mL of normalized culture was placed in a 100 mm petri dish giving a 2.5 mm depth of culture. The dish sat on packed ice while being exposed to a total of 150 J/m^2 254 nm UV light by a model UVGL-25 Minerallight® lamp by UVP, Inc. To ensure consistent exposure, the UV was measured with a Spectroline® Digital Radiometer model DRC-100X. The 20 mL culture of 0.25×10^9 cells/mL was centrifuged at 4° at 3500 g for 10 minutes into a pellet and resuspended back to the original culture concentration. The samples remained on ice or in a centrifuge at 4°C until they were concentrated and ready to be incubated. The samples were left in a 37°C shaking

water bath for varying lengths of time before the cells were pelleted, resuspended in 300 µL 4M guanidine isothiocyanate (GITC)-50 mM Tris-HCl, pH 7.0 (GITC lysis buffer) and frozen in liquid nitrogen. The samples were stored at -80 °C until the polyphosphate extraction procedure was performed.

Cisplatin Survival Assay

The survival of *E. coli* after cisplatin exposure was assayed with cells grown in MinA minimal media at 37°C to stationary phase. The concentrations of the cultures were determined using the optical density at 600nm wavelength and normalized to 0.5 x 10^9 cells/mL. 0.5 mL of normalized culture was placed in a 1.5 mL eppendorf tube. An additional 0.5 mL of MinA with cisplatin (from Sigma) was added for a final concentration of 0, 5, 10, 15, 20, 25, 30, or 40 µg/mL cisplatin. The samples were incubated at 37°C for 1 hour before being diluted in LB media. 0.1 mL aliquots were spread on LB plates and incubated overnight at 37°C. The colonies on the plates were counted and the viable cell counts were calculated.

Gamma Irradiation Survival Assay

The survival of *E. coli* after gamma irradiation was assayed with cells grown in MinA minimal media at 37°C to stationary phase. The concentrations of the cultures were determined using the optical density at 600nm wavelength and normalized to 0.25 x 10^9 cells/mL. 20 mL of normalized culture was placed in a 100 mm petri dish giving a 2.5 mm depth of culture. The dish was placed in a JL Shepherd Mark I Cesium Irradiator at room temperature. The exposure was 2.99 Gy/min and at the specified times, 0.5 mL samples were withdrawn from exposed sample and diluted in LB media. 0.1 mL aliquots were spread on LB plates and incubated overnight at 37°C. The colonies on the plates were counted and the viable cell counts were calculated.

PolyP Extraction

Polyphosphates were extracted from cell pellets using a similar method as previously described (Ault-Riche *et al.*, 1998). *E. coli* cultures were pelleted in a 1.5-mL tube and resuspended by adding 0.3 mL of 4 M guanidine isothiocyanate (GITC), 50 mM Tris-HCl, pH 7.0 (GITC lysis buffer). The sample was then either processed directly or frozen in liquid nitrogen and stored at -80°C. The sample was thawed and heated for 5 minutes at to 95°C, vortexing every minute. The samples were then placed in a Branson 2510 bath sonicator for 30 minutes at room temperature. A 25 µl sample was removed for protein estimation. 30 µl of 10% sodium dodecyl sulfate (SDS) was added to the sample and heated to 95°C for 2 minutes. 0.3 mL of 200 proof ethanol and 8 µl of Glassmilk® from MP Biomedicals (silica slurry) were added, the sample thoroughly mixed and heated for an additional 30 seconds at 95°C. The tube was centrifuged briefly to pellet the precipitate with DNA, RNA and polyP bound to the silica. The samples were then suspended in 0.2 mL of cold Wash buffer (5 mM Tris-HCl [pH 7.0], 50 mM NaCl, 5 mM EDTA, 50% ethanol) and repelleted; washing was repeated twice. The washed pellet was then resuspended in 200 µl of 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, and 20 µg each of DNaseI and RNaseA per mL and incubated at 37°C for 1 hour. (Nucleases from Roche Diagnostics) The pellet was washed twice with 200 µl Wash buffer. After the final spin and after

the remaining ethanol evaporated from the pellets leaving the sample dry, polyphosphates were eluted from the pellet by adding 25 µl of 50 mM Tris-HCl (pH 8.0) and heated to 95°C for 1 min. The sample was centrifuged and the supernatant containing the polyP was saved. The elution was repeated with an additional 25 µl of 50 mM Tris-HCl (pH 8.0) and the supernatants were pooled for a total of 50 µl of extracted polyP.

PolyP Measurement

PolyP samples were measured using a similar method as previously described (Ault-Riche *et al.*, 1998). 10 µL of polyP samples and 10 µL of reaction mixture (50 mM Tris-HCl [pH 7.4], 40 mM ammonium sulfate, 4 mM MgCl2, 5 µM ADP, 24,000 U of PPK) were incubated at 37°C for 40 min. The reaction mixture was diluted with 80 µL of 100 mM Tris-HCl (pH 8.0), 4 mM EDTA to a total of 0.1 mL. Further dilutions were performed as necessary. 50 µL were placed into a well of a black 96 well plate. 50 µL of luciferase reaction mixture (ATP Bioluminescence Assay Kit from Roche Diagnostics) were added to the well and mixed by pipeting the solution in and out twice. Luminescence was measured 10 minutes after the luciferase reaction mixture was added with a Perkin Elmer Victor^3 V 1420 multi- labeled counter. The luminometer measured each well for 10 seconds. ATP concentrations of 10^{-3} to 10^{-12} M in 100 mM Tris-HCl (pH 8.0), 4 mM EDTA were also measured to be used as a standard curve. Concentrations of polyP were calculated in terms of orthophosphate residues. Final cellular concentrations were determined by orthophosphate residues per mg of protein. Protein concentrations of the original cell samples were determined utilizing the Bradford method with Bio-Rad protein assay dye reagent and bovine serum albumin as a standard.

KEY ACCOMPLISHMENTS

Research Accomplishments

Discovered:

- Loss of ability to synthesize polyphosphates compromises cellular survival after DNA damage
- The effect is not exclusive to a specific DNA damaging agent
- The loss of survival is not directly linked to the general stress response pathway
- The compromised survival is independent of cellular growth phase or growth rate
- Polyphosphate levels increase transiently after DNA damage
- Elevated polyphosphate levels are not dependent on the SOS genes *recA*, *dinB*, or *umuDC*
- The ability to synthesize polyphosphates influences Pol IV activity
- A potential role for polyphosphates in either the initiation of Pol IV mediated error-prone DNA repair or the inhibition of other DNA repair mechanisms. (See model in the conclusion)

Developed:

- An optimized procedure to extract polyphosphates from breast cancer cells with excellent and reproducible yield
- A construction of a mammalian expression plasmid containing *S. cerevisiae* exopolyphosphatase (scPPX) gene behind a constitutively active CMV promoter
- A DNA transfection of the constructed plasmid into MCF-7 breast cancer cells
- Thirty-six possible clones of MCF-7 cells stably transfected with scPPX
- Screened half of the stable transfectants to determine the existence of scPPX in the chromosome
- Genetically modified strains used for epistatic studies to help determine the molecular mechanism of polyphosphates in DNA-damage survival
- Optimal, reproducible polyphosphate extraction and measurement procedures for *E. coli*
- Standard UV radiation procedures for *E. coli* cells

Training Accomplishments

- Participated in numerous seminars concerning breast cancer associated with the Lombardi Comprehensive Cancer Center

- Participated in the Mutagenesis Gordon Research Conference held at Salve Regina University, Newport, Rhode Island – August 6-11, 2006
- ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) meeting held in the Pennsylvania Convention Center in Philadelphia, Pennsylvania on June 8-11, 2005
- Participated in weekly seminars and weekly journal clubs that addressed not only research specific to breast cancer but also advances in other cancer research and basic science research. Awareness of other areas of research and new laboratory techniques inspire new ideas for conquering breast cancer.
- Participated in the Breast Cancer Symposium hosted by Georgetown University on December 6, 2004
- Enhanced skills in tissue culture, DNA transfection, plasmid design and development, and genetic analysis

REPORTABLE OUTCOMES

THESIS

- Haakenson,C. (2007) "Polyphosphates Enhance Cell Survival Following DNA Damage by Influencing SOS Induced DNA Repair Mechanisms", Unpublished Doctoral Dissertation, Georgetown University

MANUSCRIPTS IN PREPARATION

- Haakenson,C., Crooke, E. (2007) "Polyphosphates Enhance Survival after DNA Damage in *Escherichia coli*"

PUBLISHED ABSTRACTS

- Haakenson,C., Crooke, E. (2004) "Role of Inorganic Polyphosphates in Stress Response Pathways Enhancing Cell Survival", [Abstract], Georgetown University Student Research Days, Washington, D.C.
- Haakenson,C., Crooke, E. (2005) "Effects of Cellular Polyphosphate on Breast Cancer Cell Survival", [Abstract], Era of Hope Department of Defense Breast Cancer Research Program Meeting - Proceedings, Philadelphia, Pennsylvania
- Haakenson,C., Crooke, E. (2005) "Role of Cellular Polyphosphates in Enhancing Survival after DNA Damage", [Abstract], Georgetown University Student Research Days, Washington, D.C.
- Haakenson,C., Crooke, E. (2006) "Role of Cellular Polyphosphates in Enhancing Survival after UV Induced DNA Damage in *Escherichia coli*", [Abstract], Georgetown University Student Research Days, Washington, D.C.

- Haakenson,C., Crooke, E. (2007) "Role of Polyphosphates in Enhancing Survival after DNA Damage in *Escherichia coli*", [Abstract], Experimental Biology 2007, Washington, D.C.

PRESENTATIONS

- Mutagenesis Gordon Research Conference – "Role of Cellular Polyphosphates in Enhanced Survival after DNA Damage" August 6-11, 2006
- ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting – "Effects of Cellular Polyphosphate on Breast Cancer Cell Survival" June 8-11, 2005
- Georgetown University Department of Biochemistry and Molecular Biology Graduate Student Data Presentation – "Role of Cellular Polyphosphates in Enhancing Survival after DNA Damage in *Escherichia coli"* September 25, 2006
- Georgetown University Department of Biochemistry and Molecular Biology Graduate Student Data Presentation – "Role of Cellular Polyphosphates in Enhancing Survival after UV Induced DNA Damage in *Escherichia coli"* October 17, 2005
- Georgetown University Department of Biochemistry and Molecular Biology Graduate Student Data Presentation – "Role of Inorganic Polyphosphates in Enhancing Survival after UV Induced DNA Damage in *Escherichia coli"* November 1, 2004
- Georgetown University Biomedical Research Days Poster Exhibition – "Role of Cellular Polyphosphates in Enhancing Survival after UV Induced DNA Damage in *Escherichia coli"* February 23, 2006
- Georgetown University Biomedical Research Days Poster Exhibition – "Role of Cellular Polyphosphates in Survival in Stress Response Pathways Enhances Cell Survival*"* February 24, 2005
- Georgetown University Biomedical Research Days Poster Exhibition – "Role of Inorganic Polyphosphates in Stress Response Pathways Enhancing Cell Survival*"* February 26, 2004

CELL LINES AND STRAINS

- Construction of strains with genetic modifications to silence following genes: *ppk, recA, uvrA, polB, dinB, umuC,* double knockouts of *ppk* and each of the other genes, double knockouts of *dinB, umuC,* and triple knockout of *ppk, dinB, umuC*
- MCF-7/PPX – A new breast cancer cell line originating from MCF-7 cells with a genomic copy of *ppx* controlled by a CMV promoter

OTHER

- Application for a Susan G. Komen Breast Cancer Foundation – Basic Clinical and Transduction Grant - "Cellular Polyphosphates and Breast Cancer Response to Chemotherapeutic and Radiation Treatments" P.I. Elliott Crooke Ph.D.

CONCLUSIONS

(Large portions of the conclusion of this report is adapted from doctoral thesis: Haakenson,C. (2007) "Polyphosphates Enhance Cell Survival Following DNA Damage by Influencing SOS Induced DNA Repair Mechanisms", Unpublished Doctoral Dissertation, Georgetown University)

The recipient of this pre-doctoral training grant from the Department of Defense not only built knowledge about cell survival in relation to polyphosphates and breast cancer, but also in her training as a biochemical scientist preparing for a career in breast cancer research. Due to the novel idea of polyphosphates having a potential role in breast cancer, work toward the initial goal of the proposal to examine the physiology of breast cancer cells with altered polyphosphate levels is still ongoing. However, the work performed gained insight into the possible molecular mechanism of polyphosphates in stress survival by exploiting the emerging understanding of how polyphosphates promote survival of prokaryotes following DNA damage. The information gained from the studies described in this report will help direct the continuing examination of polyphosphates in breast cancer cell survival.

Discussion

The involvement of polyphosphates in cellular survival after DNA damage has been examined. Exposure to UV radiation triggers the SOS response. Determining how polyphosphates may be affecting SOS response pathways, and the mechanism of how this occurs, will build a better knowledge of other possible functions of polyphosphates.

From the results presented here, seven major findings can be stated:

- Loss of ability to synthesize polyphosphates compromises cellular survival after DNA damage
- This effect is not exclusive to a specific DNA damaging agent
- This loss of survival is not directly linked to the general stress response pathway
- This compromised survival is independent of cellular growth phase or growth rate
- Polyphosphates levels increase transiently after DNA damage
- Elevated polyphosphate levels are not dependent on the SOS genes *recA*, *dinB*, or *umuDC*
- The ability to synthesize polyphosphates influences Pol IV activity

Polyphosphates, or PPK, affect the SOS response

It is shown that polyphosphates enhance the viability of cells that have been exposed to UV radiation. At low doses of UV light, little difference is seen between the viability of wild type and PPK⁻ strains. However, at the elevated levels of UV, there is nearly a 10-fold difference in survival. With low levels of radiation, DNA damage triggers DNA repair, but the extent of damage is not sufficient to activate the SOS response. At these low levels, no survival difference is observed between cells with and without PPK. As the level of DNA damage increased, the SOS response is initiated, and the survival difference between PPK⁻ and wild type becomes markedly different. The effects of polyphosphates on the survival are not observed without elevated levels of DNA damage which trigger the SOS response.

Additional evidence that polyphosphate's involvement is not specific to one form of DNA repair is shown in figures 17 and 18. Double stranded breaks, caused by gamma irradiation, are not repaired by the NER system. With increased doses of gamma irradiation, cells without PPK are more susceptible to killing than wild type cells. This suggests the effect of polyphosphates on survival after DNA damage is not specific to the type of DNA lesion formed but rather to a common survival mechanism activated by the DNA damaging agents, i.e. the SOS response.

RpoS and SOS Response

While the involvement of polyphosphates in the general stress response, including their affects on *rpoS* transcription and activity (Shiba *et al.,* 1997), have been described, there is no reported connection between RpoS and the SOS response. Yet, RpoS⁻ cells have decreased viability when radiated with UV. Interestingly, the RpoS⁻ cells cannot be rescued by overproduction of polyphosphates, nor did they show decreased polyphosphate levels.

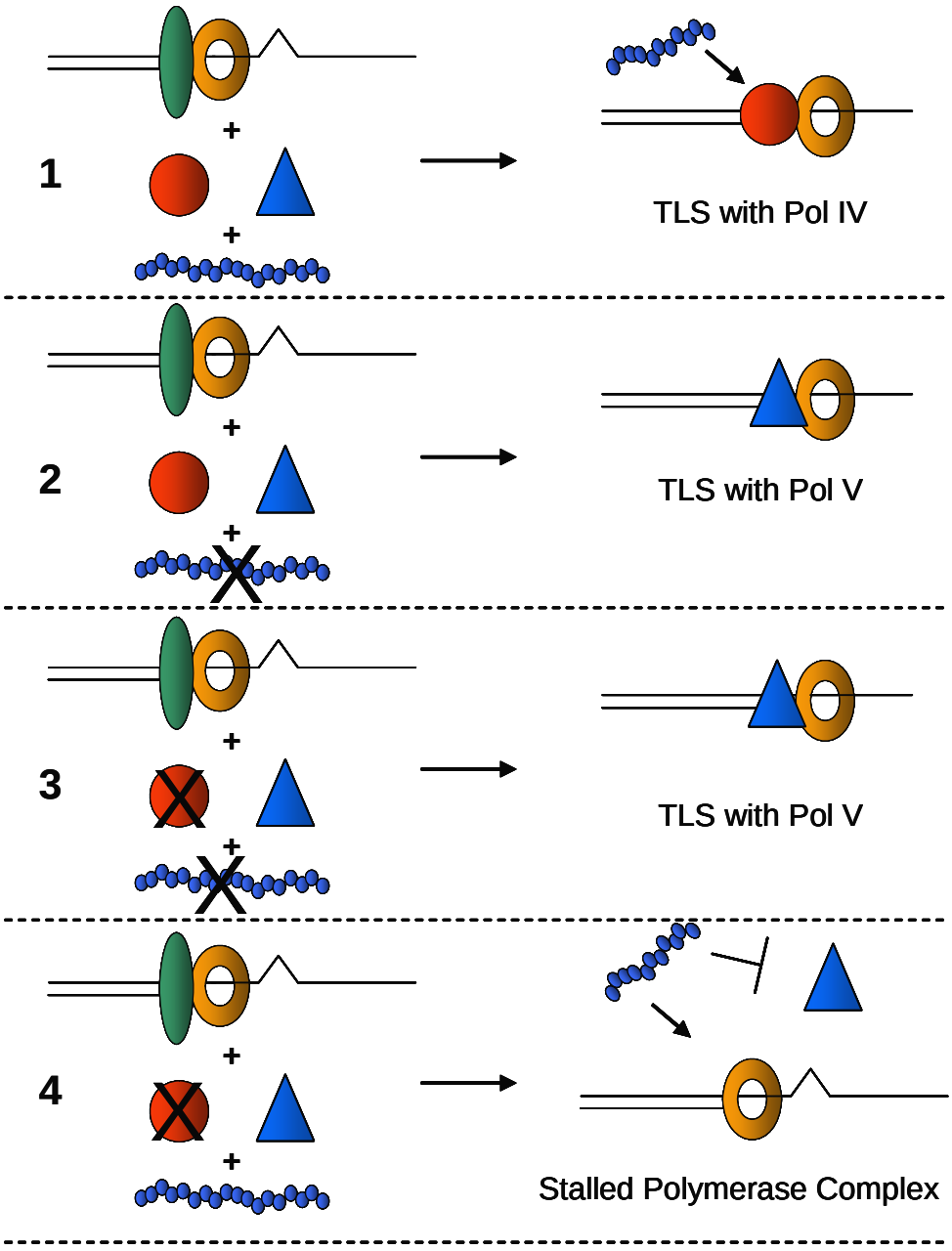
The transcriptional activation of SOS genes by LexA derepression does not require a specific sigma transcription factor. Therefore, it is possible that the effect of RpoS on cell survival may be due to ongoing utilization as the sigma factor present at the time of the DNA damage to transcribe the SOS response genes.

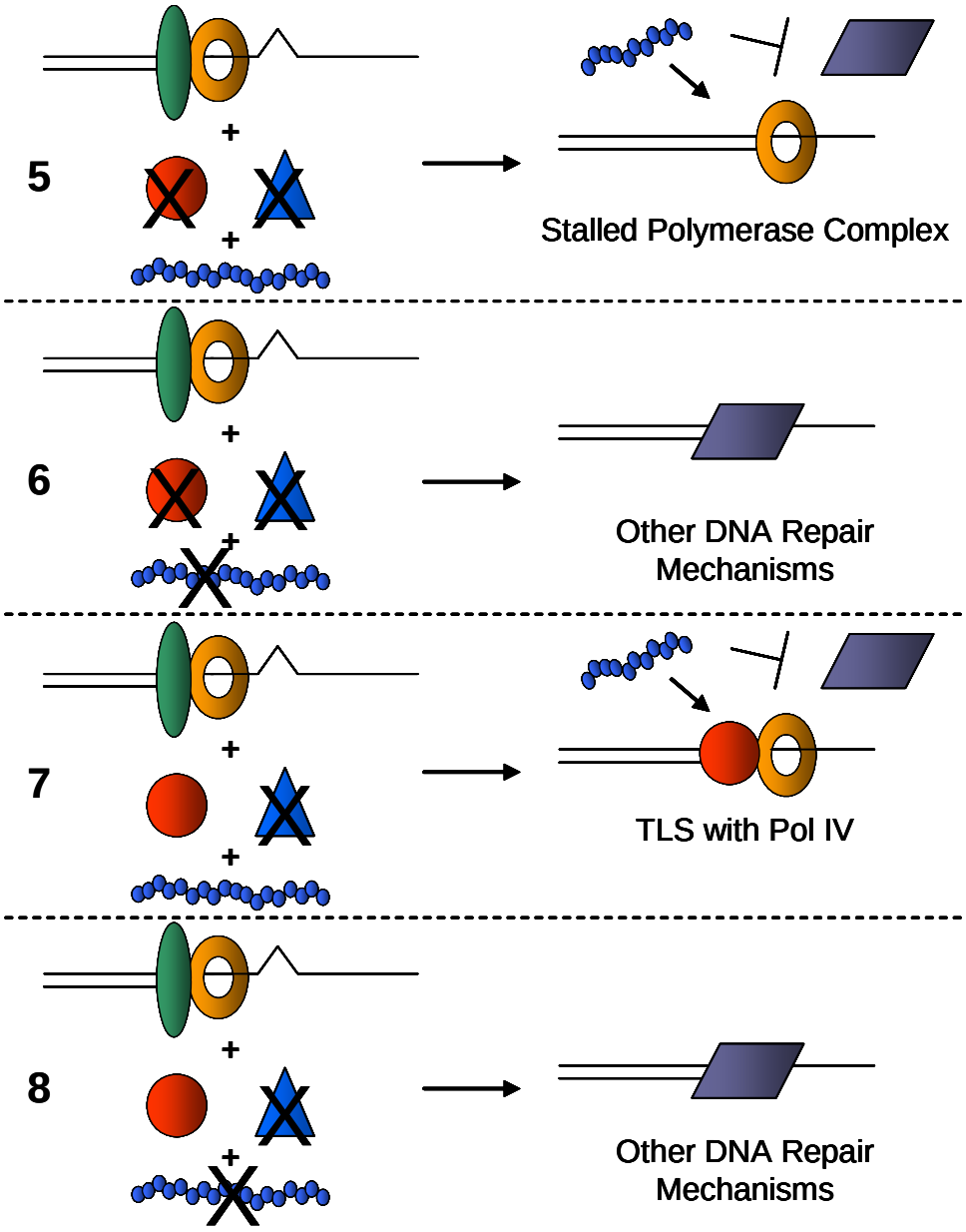
The data show that polyphosphates affect the SOS response separate from the general stress response; nevertheless, it does not imply that the activation of the general stress response could not influence the actions of polyphosphates in the SOS response.

Polyphosphates and SOS Proteins

The epistatic experiments were performed with PPK and key SOS proteins to gain mechanistic insight. PPK and RecA, as well as PPK and Pol V, show an additive effect on the sensitivity that the cells have to UV radiation, indicating polyphosphates influence the survival of cells through a different pathway, or at least partially different, than either RecA or Pol V.

Pol IV knockout cells have shown the most intriguing results. The Pol IV single knockout is more sensitive than the Pol IV and PPK double knockout, suggesting polyphosphates act upstream from the Pol IV error prone polymerase, and without polyphosphates the cells do not activate the pathway that emphasizes Pol IV TLS. The double knockout of Pol IV and Pol V proteins was very sensitive to UV, but removal of PPK to form a triple knockout restored some viability, similar to what was observed for the Pol IV$^-$ versus Pol IV$^-$/PPK$^-$ cells. In both cases when Pol IV was interrupted, the absence of PPK decreased the killing by UV radiation. To explain this situation, the following model (figure 32) was developed. It is important to note that this model does not suggest that the DNA repair occurs by an "all or nothing" mechanism. There likely is a balance between Pol IV TLS, Pol V TLS, recombination repair, NER and other DNA repair mechanisms. The model contains 8 conditions, each explained in table 3.


1
TLS with Pol IV
2
TLS with Pol V
3
TLS with Pol V
4
Stalled Polymerase Complex


5
Stalled Polymerase Complex
6
Other DNA Repair Mechanisms
7
TLS with Pol IV
8
Other DNA Repair Mechanisms

SUMMARY

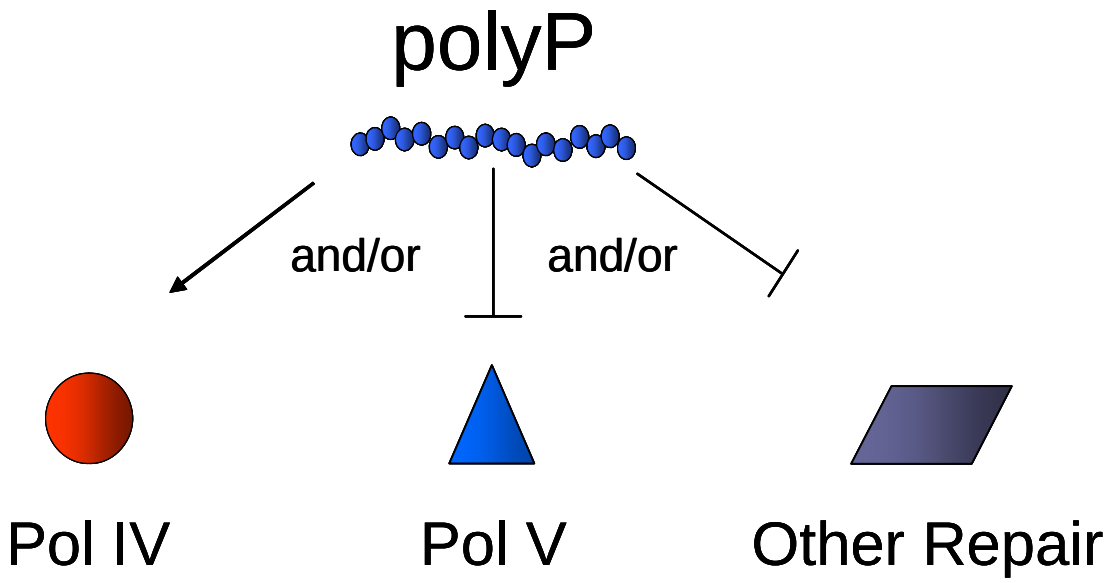


Figure 32 – Model for polyphosphates participation in TLS during the SOS response

The above model illustrates the effects polyphosphates have on the selection of DNA repair mechanism. Table 3 below describes each step.

Table 3 – Steps of the model for polyphosphates and DNA repair

	CONDITIONS	RESULTS
1	WT cells grown in MinA to stationary phase. Cells contain Pol IV, Pol V, and polyP.	All DNA repair mechanisms are functional and polyP selects a preference for Pol IV TLS.
2	PPK$^-$ cells grown in MinA to stationary phase. Cells contain Pol IV, Pol V, but not polyP.	All DNA repair mechanisms are functional but without polyP there is no longer a preference for Pol IV TLS. Pol V TLS now dominates.
3	Pol IV$^-$/PPK$^-$ cells grown in MinA to stationary phase. Cells do not have Pol IV TLS or polyP influence but they do have Pol V.	Pol IV TLS is not functional and polyP can not influence the preference to Pol IV.
4	Pol IV$^-$ cells grown in MinA to stationary phase. Cells do not have Pol IV TLS.	Pol IV TLS is not functional but polyP selects a preference for Pol IV. This is done either by inhibiting Pol V and the other repair mechanism or by initiating Pol IV repair which can not be executed.
5	Pol IV$^-$/Pol V$^-$ cells grown in MinA to stationary phase. Cells do not have Pol IV TLS or Pol V TLS.	None of the error prone repair mechanisms are functional, yet polyP selects a preference for Pol IV. This is done either by inhibiting other repair mechanisms, recombination, or by initiating Pol IV repair that can not be executed.
6	Pol IV$^-$/Pol V$^-$/PPK$^-$ cells grown in MinA to stationary phase. Cells do not have Pol IV TLS, Pol V TLS, or polyP influence.	None of the error prone repair mechanisms are functional. PolyP does not influence the repair balance, and therefore other repair mechanisms, such as recombination, can act.
7	Pol V$^-$ cells grown in MinA to stationary phase. Cells do not have Pol V TLS.	Pol IV TLS and other DNA repair mechanisms are functional, and polyP selects a preference for Pol IV TLS.
8	Pol V$^-$/PPK$^-$ cells grown in MinA to stationary phase. Cells do not have Pol V TLS or polyP influence, but they do have Pol IV.	Pol IV TLS and other DNA repair mechanisms are functional, but polyP does influence repair. Other repair mechanisms, such as recombination, perform the balance of the repair.
	SUMMARY	Polyphosphates influence the balance of the repair mechanisms by causing a preference towards Pol IV TLS. This is done by either inhibiting other forms of repair or by initiating Pol IV TLS.

A relationship between Pol IV and polyphosphates has previously been suggested (Stumpf and Foster, 2005). While looking at adaptive mutagenesis, it was discovered that interruption of the *ppk* gene caused a decreased level of mutations. Pol IV levels in the strain with an interrupted *ppk* gene remained at wildtype levels, showing that the decrease in Pol IV activity in PPK^- cells was not due to decreased levels of Pol IV protein (Stumpf and Foster, 2005). This supports the theory that polyphosphates, or PPK, influence the activity of the Pol IV replisome. There are 250-1000 molecules of Pol IV protein in the cell when the cells are not induced for SOS, and at least 2500 molecules in SOS-induced cells (Kim et al., 2001). Pol IV induction also occurs in cells during stationary phase, the growth environment of adaptive mutagenesis, and is independent of the SOS response (Layton and Foster, 2003). The induction of Pol IV generates enough Pol IV molecules to compete with other polymerases for assembly with the β-clamp at the replication forks.

The epistatic experiments presented here gave insight on the relationships between *ppk* and the SOS genes, but they did not reveal simple, conclusive relationships. Even the SOS proteins amongst themselves do not have simple relationships. For example, the RecA protein is involved in initiating the SOS response by binding to single stranded DNA and activating the autocleavage of LexA. Once LexA leaves the promoter regions, the SOS genes are transcribed, with one of these genes being RecA. The additional RecA continue to bind single stranded DNA, thus amplifing the SOS response and helping to activate UmuD for Pol V formation. RecA also participates in both recombination DNA repair and Pol V error prone repair. As the DNA is repaired, less RecA is activated by single stranded DNA, allowing LexA to inhibit DNA transcription of the SOS genes, thereby causing the SOS response to end. Therefore, RecA is activating the pathway, participating in DNA repair through multiple channels, and at the completion of the response. In fact, all SOS proteins that are involved in DNA repair participate in the pathway feedback to stop the SOS induction. Because of the complexity of the SOS response, it is not surprising the association with polyphosphates is also complex.

Model Scenarios

The model in figure 32 suggests that polyphosphates either inhibit other forms of repair, such as Pol V error prone repair and recombination repair, or initiate Pol IV TLS. One hypothesis for polyphosphates participation in the initiation of Pol IV TLS involves Pol IV and the β-clamp. Polyphosphates, directly or indirectly, may assist in the loading of Pol IV onto the β clamp. In the absence of polyphosphates, Pol IV can not be loaded for TLS, but Pol V can, and therefore still, carry out TLS. However, if polyphosphates are present without Pol IV, the β clamp is capable solely for Pol IV loading, which prevents Pol V replication and DNA repair, and thus resulting in a decrease in cell survival.

A possible explanation of how polyphosphates may inhibit other forms of repair involves RecA protein. It is believed the L2 loop of the RecA protein binds single stranded DNA on the phosphate backbone (Bell, 2005; Kelley and Knight, 1997), so it is quite possible that RecA binds polyphosphates. Polyphosphates and DNA may compete for RecA binding, regulating RecA involvement in DNA repair by Pol V and homologous recombination. Therefore with polyphosphates, repair by Pol V and recombination are reduced, but damaged DNA can be repaired by Pol IV. Without Pol IV but with polyphosphates, the Pol V and recombination repair are still reduced, but Pol IV is not able to repair the damage, decreasing survival of the cells.

Without Pol IV and without polyphosphates, the repair capability of Pol V and recombination are not limited, and cell survival is maintained. The same logic applies to the Pol V and Pol IV double knockout strains. With polyphosphates in the double knockout, recombination is still restrained, reducing survival. In contrast, without polyphosphates, recombination can proceed and survival is again maintained.

Perspectives

Polyphosphates and the Cell Status

Proteins are translated throughout the life of the cell during cell proliferation and during quiescence due to nutrient deprivation. While protein synthesis remains active, the quantity of proteins, types of proteins, and speed of translation vary based upon the status of the cell. The cellular status may also affect the SOS response. It has previously been stated that TLS bypass efficiencies *in vivo* depend on the identity of the lesion and the polymerase used to copy it; for example, Pol V is much more efficient than Pol II and Pol IV for the repair of abasic sites, TT dimers, and 6-4 photoproducts (Tang *et al.*, 2000), whereas bulky adducts such as acetyl aminofluorenes (AAFs) and benzopyrenes are better substrates for Pols II and IV. (Schlacher *et al.*, 2006b; Shen *et al.*, 2002; Wagner *et al.*, 2002). This suggests Pol IV would not have a significant effect on cell survival after UV radiation, but in figure 30 the Pol IV^{-} strain had greater sensitivity than the Pol V^{-} strain. In contrast to the studies described here, the previous studies compared Pol V and Pol IV activity following UV radiation in rich media or in log phase. This suggests that the balance of DNA repair mechanisms activated by SOS is affected by the status of the cells. Further studies using the strains developed for this research cultured under different growth conditions could test this theory.

The cellular physiology is constantly adjusted to obtain the needed balance of pH, protein concentrations, and salt concentration. Each of these affect protein-protein interactions as well as protein-nucleic interactions (Bujalowski, 2006). Polyphosphates may be part of the cellular process to balance these variables. For example, as cells enter stationary phase there is a switch in the sigma factors for RNA polymerase from σ^{70} to σ^{s}, RpoS. The binding of these two sigma factors has been found to be dependent on the salt concentrations. High concentrations of potassium glutamate, mimicking the intracellular ionic conditions under hyperosmotic stress, specifically enhanced transcription by RpoS, but inhibited that by σ^{70} (Ding *et al.*, 1995). The same group discovered that polyphosphates may play a role in the promoter selectivity control of RpoS. The RNA polymerase from the stationary growth phase cells is tightly associated with an acidic compound(s) and exhibits altered promoter selectivity, with reduced transcriptional activity of the genes highly expressed in the exponentially growing cells. When the polymerase was isolated, polyphosphates were found tightly associated. The inhibitory effects of polyphosphates on transcription were examined *in vitro* using two holoenzymes, one containing σ^{70} and the other containing RpoS. At low salt concentrations, polyphosphates inhibited transcription by both σ^{70} and RpoS holoenzymes. With an increase in the concentration of potassium glutamate, the polyphosphates inhibition was relieved. At high salt concentrations, the σ^{70} holoenzyme is not able to function, but the RpoS holoenzyme remains activated (Kusano and Ishihama, 1997). If polyphosphates help control the balance of transcription factors based

upon the cellular status, they may also help control the balance of DNA polymerases during DNA repair.

Polyphosphates versus Polyamines

Polyamines (putrescine, spermidine, and spermine) are similar to polyphosphates in many ways, even though they have opposite ionic properties. Polyamines are ubiquitous, polycationic compounds containing two or more amino groups. Although polyamines are seen to be synthesized in cells via highly regulated pathways, their functions are not entirely clear. As cations, they bind to DNA, RNA, nucleotide triphosphates, other acidic substances, and perhaps polyphosphates, though that has not been directly tested. Because polyamines can bind all of these acidic compounds, they have the capability to influence many biological processes. It is known that polyamines participate in normal cell growth and other processes such as nucleic acid biosynthesis, protein synthesis and cellular viability. But, similar to polyphosphates, the mechanism of action remains unclear (Igarashi and Kashiwagi, 2000).

Polyamines have also been found to affect the SOS response. Kim and Oh reported the effects of polyamines on *recA* gene SOS induction following exposure to UV, γ-irradiation and mitomycin C. Polyamine deficient mutant and wild type *E. coli* strains carrying *lacZ* reporters were employed to quantitatively measure variances in *recA* gene expression. In the polyamine mutant, the induction of *recA* transcript by DNA damage was up to 4-fold lower than that for wild type. The exogenous addition of polyamines restored the reduced induction of the *recA* gene to the wild type level. It is suggested that polyamines may play an essential role as a mediator of SOS induction following exposure to damaging agents in *E. coli* (Kim and Oh, 2000).

How could the disruption of polyphosphates and polyamines, two compounds of different charge, cause similar phenotypes in cell survival? Here, it is suggested that there is a sensitive balance in the cellular physiology, such as pH, salt concentrations, and protein concentrations. Part of that balance may involve both polyphosphates and polyamines. Polyphosphate levels are reported to rise upon entering stationary phase as the general stress response is activated and the cells switch from proliferation to quiescence. Therefore, polyphosphates, or polyamines, may be part of the cell's physiological modifications in adapting to a less plentiful growth condition. While polyphosphates can compete with DNA for DNA binding proteins, polyamines can bind to DNA and prevent protein binding, and both polyions are able to stabilize ionic charge variations in the cells. The two fields of research should recognize each other and build on each other's knowledge.

Polyphosphates and Protein Binding

In vitro studies have revealed that polyphosphates bind the Lon protease and ribosomal proteins (Kuroda *et al.*, 2001; McInerney *et al.*, 2006). The binding occurs within the Lon protease ATPase domain, which also binds DNA (Nomura *et al.*, 2004). This is not surprising since polyphosphates and DNA have similar chemical and structural properties. A protein that binds the phosphodiester backbone of DNA may also bind the phosphoanhydride chain of polyphosphates. If this is true, polyphosphates could have the capability to regulate numerous DNA binding proteins.

An example of a protein which binds the phosphodiester backbone of DNA is RecA. As discussed above, RecA controls the SOS response and participates in multiple mechanisms within this response pathway. If polyphosphates are also bound by RecA, the decrease in cell survival due to the loss of polyphosphate synthesis may be related.

The potential functions of polyphosphates become seemingly endless if polyphosphates influence the binding of DNA by DNA binding proteins. It has already been observed that polyphosphates may affect the binding of the sigma transcription factors as cell physiology varies (Ding *et al.*, 1995; Kusano and Ishihama, 1997). Other events polyphosphates may influence are histone binding and chromatin structure, replication, transcription, and DNA repair.

Future Directions

The evolutionary significance of polyphosphates is evident through polyphosphates' ubiquitous existence even though their synthesis entails an expense of energy. Because of this, polyphosphates likely participate in basic cellular functions required for cellular life, but to date there is very limited understanding of what these functions may be. Therefore, the potential for future studies of polyphosphates is extensive. One of the future possibilities is the translational research for breast cancer research as discussed below.

Cancer Treatment

If the function of polyphosphates in cell survival remains through evolution, polyphosphates in mammalian cells may have a role in cancer progression, and therefore be an attractive target for treatment. If cancer cells have greater content of polyphosphates, or altered functions for polyphosphates compared to normal cells, the polyphosphates may be contributing to the increased survival and proliferation of cancer cells. Therefore, the study of polyphosphates may lead to both a new cancer biomarker as well as a potential therapeutic target.

As a target, compounds could be designed to block polyphosphate synthesis within breast cancer cells. Treatment with such compounds would render the cells more susceptible to therapies that placed stress on the cells, such as chemotherapy or radiation therapy. The increased efficacy would permit cytotoxic treatments to have shorter treatment periods and lower doses resulting in less damage to healthy cells, ultimately improving the patient's quality of life.

If it is found that the cancer cells have elevated levels of polyphosphates, cellular polyphosphate concentrations could be measured as a biomarker of cancer. Future studies could investigate the correlation of polyphosphate levels and invasiveness of the cancer. If a strong correlation is found, treatment regiments could be determined based in part upon the polyphosphate levels.

Before focusing on the design of diagnostics and therapies, the basic science of polyphosphates in cancer needs to be determined. First, the polyphosphate levels in cancer cells must be compared to those in comparable non-tumorigenic cells. Altered levels of polyphosphates may lead to increased cell survival, as seen in other cellular models. Second, the

physiological effects polyphosphate depletion has on the survival of cancer cells must be determined. Currently it is not known whether polyphosphates affect mammalian cell survival following environmental stresses. Based on these results, further studies should be carried out to determine the possible molecular pathways in which polyphosphates participate.

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